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PAGE

The Action of Potassium and other Ions on the Injury Potential and Action Current in *Maia* Nerve. By S. L. Cowan. Communicated by A. V. Hill, F.R.S. 216

Physico-Chemical Studies of Complex Organic Molecules. Part I.—Monochromatic Irradiation. By F. P. Bowden and C. P. Snow. Communicated by T. M. Lowry, F.R.S. (Plates 7 and 8) 261

Physico-Chemical Studies of Complex Organic Molecules. Part II.—Absorption Spectra at Low Temperatures. By F. P. Bowden and S. D. D. Morris. Communicated by T. M. Lowry, F.R.S. (Plate 9) 274

Factors influencing the Growth of Normal and Malignant Cells in Fluid Culture Media. By R. J. Ludford. Communicated by J. A. Murray, F.R.S. (Plates 10–14) 278

No. B 794—July 2, 1934.

The Limit of High Flying when Breathing Oxygen. By Sir Leonard Hill, F.R.S.... 298

The Rhythm of the Heart Beat. I—Location, Action Potential, and Electrical Excitability of the Pacemaker. By J. C. Eccles and H. E. Hoff. Communicated by Sir Charles Sherrington, F R.S. (Plate 15) 307

The Rhythm of the Heart Beat. II—Disturbance of Rhythm produced by Late Premature Beats. By J. C. Eccles and H. E. Hoff. Communicated by Sir Charles Sherrington, F.R.S........ 327

The Rhythm of the Heart Beat. III—Disturbance of Rhythm produced by Early Premature Beats. By J. C. Eccles and H. E. Hoff. Communicated by Sir Charles Sherrington, F.R.S........ 352

The Oxygen to Iron Ratio of Oxychlorocruorin and the Total Quantity of Oxygen carried by the Pigment in *Spirographis*. By H. M. Fox. Communicated by J. Stanley Gardiner, F.R.S........ 368

The Decomposition of Sodium Formate by *Bacterium coli* in the presence of Heavy Water. By A. Farkas, L. Farkas and J. Yudkin. Communicated by E. K. Rideal, F.R.S. 373

Unco-ordinated Contractions caused by Egg White and by alterations in the Cation ratio of the Medium in the Heart of the Chick embryo *in vitro*. By P. D. F. Murray. Communicated by Sir Henry Dale, Sec. R.S. 380

Hypophysectomy of Birds. I—Technique, with a Note on Results. By R. T. Hill and A. S. Parkes, F.R.S. (Plate 16)........ 402

No. B 795—August 1, 1934.

Induction of Fertility and Pregnancy in the Anoestrous Ferret. By M. Allanson, I. W. Rowlands and A. S. Parkes, F.R.S. (Plates 17 and 18)........ 410

The Habits and Structure of *Pseudapocryptes lanceolatus*, a fish in the First Stages of Structural Adaptation to Aerial Respiration. By B. K. Das. Communicated by E. W. MacBride, F.R.S. (Plates 19 and 20) 422

PAGE

The Relative Aetiological Importance of Birth Order and Maternal Age in Mongolism. By L. S. Penrose. Communicated by J. B. S. Haldane, F.R.S. 431

The Kinetics of Hæmoglobin. IV.—General Methods and Theoretical Basis for the Reactions with Carbon Monoxide. By F. J. W. Roughton. Communicated by H. Hartridge, F.R.S. 451

The Kinetics of Hæmoglobin. V.—The Combination of Carbon Monoxide with Reduced Hæmoglobin. By F. J. W. Roughton. Communicated by H. Hartridge, F.R.S. 464

The Kinetics of Hæmoglobin. VI.—The Competition of Carbon Monoxide and Oxygen for Hæmoglobin. By F. J. W. Roughton. Communicated by H. Hartridge, F.R.S. 473

The Kinetics of Hæmoglobin. VII.—Some Notes on the Reactivity of Freshly Reduced Hæmoglobin. By F. J. W. Roughton. Communicated by H. Hartridge, F.R.S. 495

The Projection of the Forebrain on the Pons and Cerebellum. By A. A. Abbie. Communicated by G. Elliot Smith, F.R.S. (Plates 21–28) 504

The Numbers of Bacterial Cells in Field Soils, as Estimated by the Ratio Method. By H. G. Thornton and P. H. H. Gray. Communicated by Sir John Russell, F.R.S. Appendix by R. A. Fisher, F.R.S. 522

Index 545

PROCEEDINGS OF
THE ROYAL SOCIETY.

SECTION B.—BIOLOGICAL SCIENCES.

615 . 778 . 547 . 831 : 6

The Antiseptic Properties of Further Amino Derivatives of Styryl and Anil Quinoline.

By C. H. BROWNING, F.R.S., J. B. COHEN, F.R.S., K. E. COOPER, S. ELLINGWORTH, and R. GULBRANSEN.

(From the Medical School, Leeds, and the Pathological Department of the University and Western Infirmary, Glasgow.)

(Received November 8, 1933.)

The antiseptic properties of a series of quaternary salts of 2(*p*-amino anil *or* styryl) quinoline have already been described (Browning and others, 1926, 1928); the present communication is a continuation of this work. The same methods have been used as before, since with compounds of the present series also the lethal action on bacteria proceeds slowly (Browning, 1922, 1926). Table I shows the inhibitory concentration of the various substances, *i.e.*, the lowest concentration which suffices to inhibit growth, so that when present in the medium inoculated with the particular organism no definite turbidity to the naked eye develops after 48 hours at 37° C.—subculture then proving sterile or yielding at most a very scanty growth. As has been noted, this concentration appears to yield the most satisfactory indication of the antiseptic effect of a slowly acting bactericidal substance.

2(p-*amino anil*)-6 *acyl amino quinoline derivatives.*—Nos. 376, 374, 373, 375, 384, and 379 are analogues of 2(*p*-amino anil)-6 acetylamino quinoline methochloride (No. 45). They are all fairly powerful antiseptics toward both staphylococcus and *B. coli* and they are little or not at all diminished in their action by serum. The members of the series show no noteworthy progressive differences in antiseptic properties which can be attributed to alterations in the acyl group. The chloracetyl compound (No. 239) is, however, less active

Table 1.

No.	Substance.	Antiseptic action.				Precipitation.	
		Staphylococcus.		*B. coli.*			
		P.	S.	P.	S.	P.	S.
376	$p\text{-NH}_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COC}_2\text{H}_5}^{6}(\text{MeCl})$	20	40	10	100	4	—
374	$p\text{-NH}_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COC}_3\text{H}_7}^{6}(\text{MeCl})$	40	40	40	100	1	—
373	$p\text{-NH}_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COC}_4\text{H}_9}^{6}(\text{MeCl})$	40	40	40	100	2	—
375	$p\text{-NH}_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COC}_5\text{H}_{11}}^{6}(\text{MeCl})$	100	100	40	100	2	—
384	$p\text{-NH}_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{CO(CH}_2)_2\text{CH}\left\langle\begin{matrix}\text{CH}_3\\\text{CH}_3\end{matrix}\right.}^{6}(\text{MeCl})$	400	100	100	100	2	—
379	$p\text{-NH}_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COCH}\left\langle\begin{matrix}\text{CH}_3\\\text{CH}_2\,.\,\text{CH}:\text{CH}_2\end{matrix}\right.}^{6}(\text{MeCl})$	100	100	20	40	10	—

239	$p\text{-}\mathrm{NH_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COCH_2Cl}}^{6}\mathrm{(MeCl)}$	100	10	20	4	4	2
383	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,CO(CH_2)_2\,.\,CH}\left\langle\begin{matrix}\mathrm{CH_3}\\ \mathrm{CH_3}\end{matrix}\right.}^{6}\mathrm{(MeCl)}$	1000	400	400	200	20	4
425	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COCH_2\,.\,CH}\left\langle\begin{matrix}\mathrm{CH_2\,.\,CH_3}\\ \mathrm{CH_3}\end{matrix}\right.}^{6}\mathrm{(MeCl)}$	1000	200	400	100	40	2
378	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COCH}\left\langle\begin{matrix}\mathrm{CH_3}\\ \mathrm{CH_2\,.\,CH:CH_2}\end{matrix}\right.}^{6}\mathrm{(MeCl)}$	400	200	400	400	10	1
334	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COC_6H_{13}}}^{6}\mathrm{(MeCl)}$	4000	400	400	200	10	—
350	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COC_7H_{15}}}^{6}\mathrm{(MeCl)}$	2000	400	400	400	10	—
351	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COC_8H_{17}}}^{6}\mathrm{(MeCl)}$	2000	400	100	200	10	—
352	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COC_{10}H_{19}}}^{6}\mathrm{(MeCl)}$	1000	200	200	200	—	—
353	$p\text{-}\mathrm{N(CH_3)_2\overset{2}{A}\,.\,Q\,.\,}\overbrace{\mathrm{NH\,.\,COC_{11}H_{23}}}^{6}\mathrm{(MeCl)}$	1000	400	200	200	10	—

Table I—(continued).

No.	Substance.	Antiseptic action.				Precipitation.	
		Staphylococcus		*B. coli.*			
		P.	S.	P.	S.	P.	S.
452	$p\text{-N(CH}_3)_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COCH}_2\,.\,\text{CH}_2\,.\,\text{COOC}_2\text{H}_5}^{6}(\text{MeCl})$	200	40	200	100	4	4
453	$p\text{-N(CH}_3)_2\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{CO[CH}_2]_4\,.\,\text{COOC}_2\text{H}_5}^{6}(\text{MeCl})$	1000	100	1000	400	10	10
447	$p\text{-NH}_2\overset{2}{\text{St}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COCH}_2\,.\,\text{CH}_2\,.\,\text{COOC}_2\text{H}_5}^{6}(\text{MeCl})$	200	20	20	40	10	40
449	$p\text{-NH}_2\overset{2}{\text{St}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{CO[CH}_2]_4\,.\,\text{COOC}_2\text{H}_5}^{6}(\text{MeCl})$	200	20	100	40	10	10
380	$p\text{-NH(C}_2\text{H}_5)\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COCH}_3}^{6}(\text{MeCl})$	400	400	100	400	4	1
292	$p\text{-NH(C}_3\text{H}_7)\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COCH}_3}^{6}(\text{MeCl})$	400	100	400	400	4	4
293	$p\text{-NH}\left(\text{CH}\begin{smallmatrix}\text{CH}_3\\ \text{CH}_3\end{smallmatrix}\right)\overset{2}{\text{A}}\,.\,\text{Q}\,.\,\overbrace{\text{NH}\,.\,\text{COCH}_3}^{6}(\text{MeCl})$	400	100	200	400	2	—

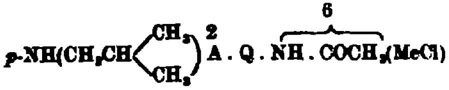
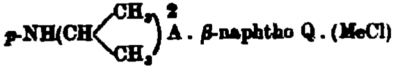
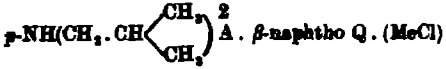
287	p-NH(C$_4$H$_9$)$\overset{2}{\text{A}}$. Q . $\overbrace{\text{NH . COCH}_3}^{6}$(MeCl)	1000	100	400	200	10	—
290	 p-NH$\left(\text{CH}_2\text{CH}\left\langle\begin{matrix}\text{CH}_2\\ \text{CH}_2\end{matrix}\right.\right)\overset{2}{\text{A}}$. Q . $\overbrace{\text{NH . COCH}_3}^{6}$(MeCl)	400	200	200	200	—	—
273	p-NH(CH$_3$)$\overset{2}{\text{A}}$. Q . $\overbrace{\text{NH . COCH}_2\text{Cl}}^{6}$(MeCl)	100	20	20	20	10	—
396	p-NH . COCH$_3$$\overset{2}{\text{A}}$. Q . $\overbrace{\text{N(CH}_3)_2}^{6}$(MeCl)	20	20	20	40	2	—
361	p-NH(C$_2$H$_5$)$\overset{2}{\text{A}}$. β-naphtho Q . (MeCl)	400	400	400	400	10	—
291	p-NH(C$_3$H$_7$)$\overset{2}{\text{A}}$. β-naphtho Q . (MeCl)	1000	200	400	200	10	—
294	 p-NH$\left(\text{CH}\left\langle\begin{matrix}\text{CH}_2\\ \text{CH}_2\end{matrix}\right.\right)\overset{2}{\text{A}}$. β-naphtho Q . (MeCl)	1000	400	200	400	4	—
286	p-NH(C$_6$H$_5$)$\overset{2}{\text{A}}$. β-naphtho Q . (MeCl)	1000	200	1000	200	10	—
289	 p-NH$\left(\text{CH}_2 . \text{CH}\left\langle\begin{matrix}\text{CH}_2\\ \text{CH}_2\end{matrix}\right.\right)\overset{2}{\text{A}}$. β-naphtho Q . (MeCl)	1000	100	200	100	4	—
296	p-CH$_3$O $\overset{2}{\text{A}}$. β-naphtho Q(MeCl)	100	10	2	20	2	—
328	m-N(CH$_3$)$_2$$\overset{2}{\text{A}}$. β-naphtho Q(MeCl)	20	40	<1	1	2	—
323	p-p dianil . β-naphtho Q(MeCl)	200	200	20	100	100	(5—)

Table I—(continued).

No.	Substance.	Antiseptic action.				Precipitation.	
		Staphylococcus.		*B. coli.*			
		P.	S.	P.	S.	P.	S.
324	m-m dianil . β-naphtho Q(MeCl)	2	10	1	4	10	—
126	p-N(CH$_3$)$_2\overset{2}{\text{St}}$. β-naphtho Q . $\overset{8}{\text{NH}_2}$(MeCl)	1000	400	<1	40	10	—
231	p-NH$_2\overset{2}{\text{St}}$. β-naphtho Q . $\overset{8}{\text{NH}_2}$(MeCl)	200	200	<1	20	10	—
249	p-NH(CH$_3$CO) . $\overset{2}{\text{St}}$. β-naphtho Q . $\overset{8}{\text{NH}_2}$(MeCl)	<2	20	<2	<2	8	4
251	p-NH(CH$_3$CO)$\overset{2}{\text{St}}$. β-naphtho Q . $\overbrace{\text{NH(CH}_3\text{CO)}}^{8}$(MeCl)	<1	1	<1	<1	4	1
325	p-N(CH$_3$)$_2\overset{2}{\text{St}}$. β-naphtho Q . (diMeCl)	2	10	<1	20	—	—
128	p-NH(CH$_3$CO)$\overset{2}{\text{St}}$. Q . $\overbrace{\text{N(CH}_3\text{)}_2}^{7}$(MeCl)	100	100	2	4	10	10
274	p-NH$_2\overset{2}{\text{St}}$. Q . $\overbrace{\text{NH(COCH}_2\text{Cl)}}^{6}$(MeCl)	10	10	<1	4	10	10
275	p-NH(CH$_3$)$\overset{2}{\text{St}}$. Q . $\overbrace{\text{NH(COCH}_3\text{)}}^{6}$(MeCl)	40	40	<1	20	—	—

120	$p\text{-NH}_2 . \overset{2}{\text{St}} . \text{Q} . \overbrace{\text{NH} . \text{CO} . \text{OCH}_3}^{6}(\text{MeCl})$	20	40	<1	4	4	2
121	$p\text{-NH}_2 . \overset{2}{\text{St}} . \text{Q} . \overbrace{\text{NH} . \text{CO} . \text{OC}_2\text{H}_5}^{6}(\text{MeCl})$	40	40	<1	10	10	—
125	$p\text{-N(CH}_3)_2\overset{2}{\text{St}} . \text{Q} . \overbrace{\text{NH} . \text{CO} . \text{OCH}_3}^{6}(\text{MeCl})$	200	200	40	100	20	—
216	$p\text{-N(CH}_3)_2 . \overset{2}{\text{St}} . \text{Q} . \overbrace{\text{NH} . \text{CO} . \text{OC}_2\text{H}_5}^{6}(\text{MeCl})$	400	200	10	100	10	—

P = medium consisting of 0·7% neutral peptone water. S = ox serum previously heated at 56° C.

Antiseptic Action.—The numbers are the reciprocals ÷ 1000, of the concentrations which suffice to produce in 48 hours at 37° C. inhibition of growth, so that the medium remains unclouded or shows at most very faint turbidity. In the former case subculture may yield no growth or, as in the latter case, may show very slight growth.

Precipitation.—The numbers are the reciprocals ÷ 1000, of the lowest concentrations which cause precipitation in the media.

Method.—The substance to be tested, in a volume not exceeding 0·1 c.c., was added to 1 c.c. of the culture medium, which consisted (*a*) of a watery solution containing 0·35% sodium chloride, and 0·7% bacteriological peptone, the hydrogen-ion concentration of the mixture being adjusted by the addition of caustic soda to yield a p_H value between 7·2 and 7·8 as indicated by the usual indicators; and (*b*) sterile ox serum, which had been previously heated for several hours at 56° C. The organisms employed in the tests were *Staphylococcus aureus* and a single strain of *B. coli*; the inoculation dose being 0·1 c.c. of a 1 : 1000 dilution in saline of a 24 hours' culture in peptone water. The mixtures were incubated at 37° C. for 48 hours, and then the final readings were made; the presence or absence of living organisms was always decided by subculturing from the mixtures on to nutrient agar, which was then incubated for 48 hours at 37° C.

in serum, especially toward staphylococcus, and this corresponds with what has been found with the chloracetyl and bromacetyl derivatives of 2(*p*-dimethylamino anil)-6 amino quinoline (*cf.* Nos. 71, 72, and 59).

2(p-*dimethylamino anil* or *styryl*)-6 *acyl amino quinoline derivatives.*—The anils (Nos. 383, 425, 378, 334, 350, 351, 352, 353, 452, 453) are higher analogues of 2(*p*-dimethylamino anil)-6 acetyl amino quinoline methochloride (No. 59). Like the other members of this series (Nos. 65–69), they are all powerfully antiseptic toward both staphylococcus and *B. coli*, and their action undergoes little or no diminution in the presence of serum. The styryls (Nos. 447, 449) are as usual less powerful than the corresponding anils (Nos. 452, 453).

2(p-*alkylamino anil*)-6 *acyl amino quinoline derivatives.*—Nos. 380, 292, 293, 287, 290 are analogues of No. 116; they are all powerfully antiseptic. Thus their action is in degree more close to that of the corresponding tertiary base (*cf.* Nos. 380 and 64), than of the primary base (No. 45), which is much weaker. The chloracetyl derivative of 2(*p*-methylamino anil)-6 amino quinoline (No. 273) is less powerfully antiseptic than the corresponding acetyl compound (No. 116)—a result which accords with previous observations on the depressing effect of a halogen atom in the acyl group (see above).

2(p-*acetylamino anil*)-6 *dimethylamino quinoline methochloride* (*No.* 396), as compared with the non-acetylated amino analogue (No. 46) shows the tendency to reduced action following acetylation of the amino group in the 2 position of the benzene nucleus which we have previously found.

2(p-*alkylamino anil*)-β-*naphthoquinoline derivatives.*—Like both the dimethylamino (No. 52) and the methylamino (No. 115) compounds, the analogues of the latter (Nos. 381, 291, 294, 286, 289) are all powerful antiseptics.

The compound with *methoxy*, instead of a basic group, in the 2 position of the benzene nucleus (No. 296) is much less active.

2(m-*dimethylamino anil*)-β-*naphthoquinoline methochloride* (*No.* 328) is weak in its action. This is in striking contrast to the corresponding *para* analogue (No. 52). It should be noted that in the *meta* compound the alternate double linkage is interfered with. Similarly with the *dianils of* β-*naphthoquinoline*, the 2-*pp* (No. 323) derivative is very active as compared with the corresponding 2-*mm* compound (No. 324).

2(p-*amino styryl*)-8 *amino* β-*naphthoquinoline derivatives.*—2(*p*-dimethylamino-styryl)-8 amino β-naphthoquinoline methochloride (No. 126) is powerfully antiseptic toward staphylococcus and so resembles in its action No. 17, which lacks the amino group in the naphthoquinoline nucleus, although it is less active toward *B. coli* in watery medium than the latter. The substitution of a primary amino (No. 231) for the dimethylamino group in the benzene nucleus has the usual effect of reducing antiseptic action, which is still further depressed by acetylation either of the amino group in the benzene nucleus (No. 249) or of both amino groups (No. 251).

2(p-*dimethylamino styryl*)-β-*naphthoquinoline dimethochloride* (*No.* 325) shows a great reduction in potency as compared with the mono-methochloride, No. 17.

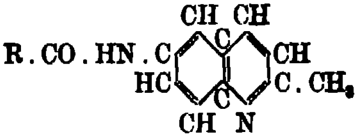
2(p-*amino styryl*)-*amino quinoline derivatives.*—2(*p*-acetylamino styryl)-7 dimethylamino quinoline methochloride (No. 128) does not differ significantly in its action from the corresponding 6 derivative, No. 90, both being fairly active toward staphylococcus. 2(*p*-amino styryl)-6 chloracetylamino quinoline methochloride (No. 274) is, like the 6 acetylamino analogue (No. 8), extremely weak. 2(*p*-methylamino styryl)-6 acetylamino quinoline methochloride (No. 275) is more powerful than the primary amino analogue (No. 8) and approaches the teritary amino compound (No. 24) in its action; this confirms other observations on the influence of the secondary amino group.

Urethane styryl derivatives.—2(*p*-amino styryl) quinolyl (6) urethane (methyl ester) methochloride (No. 120) and the corresponding ethyl ester (No. 121) are, like the 6 acetylamino analogue (No. 8), weakly antiseptic but are slightly more active than the latter. As compared with these, the 2(*p*-dimethylamino styryl) quinolyl urethanes (Nos. 125 and 216) show the usual increase in effectiveness owing to the substitution of the primary by a tertiary amino group in the benzene nucleus. There is no significant difference between the action of the methyl and the ethyl esters, as was also noted for the anil urethane derivatives (Nos. 95 and 97).

Chemical Section.

Nitroso Compounds.—These were prepared from the corresponding nitrosamines, as previously described (Browning and others, 1928). The following have not previously been prepared: p-*nitroso* iso-*propylaniline*, m.p. 86° C.; p-*nitrosobutylaniline*, m.p. 60° C.; p-*nitrosophenylethyl aniline*—an oil; p-*nitrosobenzylaniline*, m.p. 35° C.

Acylamino quinaldines.—The general method of treating the aminoquinaldine in acetic acid solution with the acid chloride (Browning and others, 1926) gave products some of which were contaminated with acetylamino quinaldine; so the method adopted for the preparation of acetyl-lactyl-aminoquinaldine, in which chloroform is substituted for acetic acid as solvent, was used (Browning and others, 1932). If precipitation of amino quinaldine hydrochloride tended to occur during the reaction, pyridine was added, and the subsequent procedure was that adopted for acetyl-lactyl-aminobenzaldehyde, as described in the same paper. These substances have the following general formula



Quaternary Salts.—Where the preparation of the methosulphate was difficult or where sodium chloride precipitated unchanged methyl *p*-toluenesulphonate from a solution of that salt, the methochloride was synthesized *via* the methiodide by the action of silver chloride in boiling alcoholic suspension. The methiodides were prepared by heating the corresponding bases with an excess of methyl iodide at 100° C. in a sealed tube.

Mono-alkyl amino anil quinoline methohalides. RHN . C_6H_4N : CH . C_9H_6N . CH_3X.—The general method has been previously described (Browning and others, 1924); occasionally as mentioned below, modifications in the method of isolation were found necessary. The physical properties of these compounds correspond roughly with those of the anils previously described. The solubility in water tended to decrease with increasing molecular weight. Generally anil condensations with *p*-acetylamino quinaldine methochloride gave a larger yield and a more crystallizable product than those with β-naphthoquinaldine methochloride.

Condensation of p-*nitroso butylaniline with β-naphthoquinaldine methochloride,* when carried out in about 70% methyl alcoholic solution yielded a crystalline product which was washed with methyl alcohol and ether and dried.

Condensation of p-*nitrosophenylethylaniline with* p-*toluquinaldine methochloride.*—When this was performed in the usual way the product did not crystallize on cooling. A concentrated solution of potassium iodide was therefore added, causing the separation of the methiodide in a somewhat tarry form, which, however, solidified on standing. After filtration it was recrystallized from alcohol and finally converted into the methochloride by boiling with silver chloride in methyl alcoholic solution.

Succinyl ester 6 amino quinaldine. $RO . CO . CH_2 . CH_2CONH . \overset{6}{C_9}H_5\overset{1}{N} . \overset{2}{CH_3}$—The ethyl hydrogen succinate was prepared by the method of Blaise (1899).

It was converted into the ester chloride by heating gently on the steam bath with an equal weight of thionyl chloride until hydrogen chloride ceased to be evolved. The excess of thionyl chloride was removed *in vacuo*, From 7 gm. of acid ester, 5·5 gm. of chloride were obtained. Succinyl ester chloride (5·5 gm.) was slowly added to 6 amino quinaldine (6·3 gm.) suspended in chloroform (40 c.c.). Heat was evolved and the base dissolved. The solution was heated for 1 hour on the steam bath, and the yellow solution filtered if necessary. The chloroform was removed on the steam bath and the clear yellow aqueous solution precipitated with sodium carbonate. The precipitate was filtered off, washed, dried, and crystallized from alcohol in the form of colourless prisms with m.p. 155°–156° C. (yield 4 gm.). It was converted into the methiodide by heating with methyl alcohol and methyl iodide in a sealed tube for 6 hours; the resulting brown crytalline crusts (2·2 gm.) were crystallized from dilute alcohol. It formed pale yellow needles which melted at 190°–192° C. (decomp.).

Found, I = 29·97%: $C_{17}H_{21}N_2O_3I$ requires I = 29·67%.

2(p-*dimethylamino styryl) succinyl ester* 6 *amino quinoline methiodide.*
4 1 2 1 6
$(CH_3)_2N.C_6H_4CH{:}CH(C_9H_5N)NH.CO.CH_2.CH_2.COOC_2H_5(CH_3I)$.—The quinaldine methiodide (1·4 gm.) was converted into the styryl compound by heating in alcoholic solution with dimethylaminobenzaldehyde (0·6 gm.) and a drop of piperidine. The product (1·6 gm.) crystallized in dark brown clusters of needles. It crystallized from hot water in flat prisms with a steely lustre and green reflex. Because of its insolubility in cold water the methiodide (1·4 gm.) was converted into the methoacetate by boiling in methyl alcohol solution with a slight excess of silver acetate (0·5 gm.) for several hours, filtered free from silver iodide and the solution evaporated on the water bath. The product (0·8 gm.) did not appear to crystallize. It formed a nearly black, hard residue, which dissolved readily in water, yielding a brown solution.

Found, I = 22·24%: $C_{26}H_{30}N_3O_3I$ requires I = 22·71%.

6 1 2
Adipic ester amino quinaldine. $C_2H_5O.CO(CH_2)_4CONH.C_9H_5N.CH_3$.—The acid ester was prepared by a method for which we are indebted to Professor Morgan. Adipic acid (29·2 gm.), absolute alcohol (38 c.c.) and concentrated sulphuric acid (4 c.c.) were heated on the steam bath for 8–10 hours; the mixture was then diluted with water (250 c.c.) and extracted with ether. The extract was shaken with excess of sodium bicarbonate solution and the aqueous layer removed, acidified, and extracted thoroughly with ether. The

ethereal layer was removed and dried. The yield from 14·6 gm. of adipic acid was 4·5 gm. It was converted into the ester chloride by warming the acid ester (4·4 gm.) with thionyl chloride (4·0 gm.) on the steam bath, as described for the succinyl ester derivative, and formed a colourless viscid liquid (yield 4·4 gm.). This was converted into the 6 adipyl ester amide of quinaldine in the manner already described. The yield was 5 gm. of recrystallized product, which consisted of long square-ended prisms, m.p. 93°–95° C. It was converted into the methiodide in the usual way. From 5 gm. of the ester amide, 3·6 gm. of orange, crystalline methiodide were obtained, which was recrystallized from boiling water. It melted at 158°–159° C. without decomposition.

Found, I = 27·89%: $C_{19}H_{25}N_2O_3I$ requires I = 27·85%.

2 (p-*dimethylamino styryl) adipyl ester* 6 *amino quinoline methiodide.*

4 1 2 1 6

$(CH_3)_2N.C_6H_4CH{:}CH(C_9H_5N)NH.CO(CH_2)_4.COOC_2H_5(CH_3I)$.—The methiodide (2·5 gm.) and dimethylaminobenzaldehyde (0·8 gm.) were heated on the water bath in alcoholic solution with a drop of piperidine. The product (1 gm.) consisted of green iridescent crystals, which dissolved in hot water with a magenta colour.

Found, I = 21·51%: $C_{28}H_{34}N_3O_3I$ requires I = 21·63%.

A portion was converted into the methochloride in the usual way. It is very soluble in water, from which it crystallizes in small prisms.

2(p-*dimethylamino anil) succinyl ester* 6 *amino quinoline methiodide.*—Succinyl ester amino quinaldine (0·8 gm.) and nitroso dimethylaniline (0·3 gm.) when condensed in the usual way gave a mass of small green iridescent crystals, which were filtered, washed with absolute alcohol, and dried. It formed clusters of dark purple needles when crystallized from hot water (yield 0·8 gm.).

Found, I = 22·36%: $C_{25}H_{29}N_4O_3I$ requires I = 22·35%. This compound is not very soluble in cold water and was converted into the methochloride in the usual way. The latter dissolved readily, forming a bright magenta solution. It crystallized from alcohol in small purple needles having a green reflex. The above and the following have the general formula,

$$(CH_3)_2N\,.\,C_6H_4N : CH(C_9H_5N)NH\,.\,CO(CH_2)_n\,.\,COOC_2H_5\,.\,(CH_3I).$$

2(p-*dimethylamino anil) adipyl ester* 6 *amino quinoline methiodide.*—The substance was prepared as above. From 0·98 gm. methiodide of adipyl ester amino quinoline and 0·3 gm. nitroso dimethylaniline, 0·8 gm. of the anil was obtained, which separated from the solution as fine purple needles with green reflex. The substance was somewhat soluble in cold water and fairly soluble n hot water with a bright purple colour.

Found, I = 21·1%: $C_{27}H_{23}N_4O_2I$ requires I = 21·31%. The methochloride was prepared from the methiodide and is readily soluble in water. It crystallizes in fine purple needles and dissolves in water with a purple colour.

Condensation of β*-naphthoquinoline methochloride 2 aldehyde with* p*-phenylene diamine and* m*-phenylenediamine.*—β-naphthoquinoline methochloride 2 aldehyde (prepared by boiling 2 gm. of 2(*p*-dimethyl amino anil) β-naphthoquinoline for 2 hours in 10 c.c. concentrated hydrochloric acid) was condensed with one-sixth of its weight of the phenylene diamine in aqueous solution by heating on the water bath (*p*-compound 2 hours, *m*-compound 5 hours). On cooling the resulting crystals were recrystallized from 64 o.p. alcohol for the *meta* product, and for the *para* compound extracted with a little alcohol to remove a reddish blue impurity.

Found, *para* compound N = 9·3%, *meta* compound N = 9·5%. $C_{36}H_{30}N_4Cl_2$ requires N = 9·5%. Both products were brownish yellow crystals.

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Effect of Absence of Light on the Breeding Season of the Ferret.

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(From the National Institute for Medical Research, London, N.W.3.)

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[Plate 1.]

I. *Introduction.*

Bissonnette's (1932) remarkable discovery that additional illumination would induce œstrus in anœstrous ferrets has naturally led to much speculation as to what controls the onset of the breeding season in the normal ferret. An obvious interpretation of Bissonnette's results, and one apparently favoured by Marshall (1932), suggested that the beginning of the breeding season of the normal female in March-April is influenced by the increasing duration of daylight. Such a hypothesis, however, could not be extended to many mammals. Those breeding in the autumn (sheep, for instance) would require a radical alteration of the hypothesis, while nocturnal and subterranean mammals can hardly be affected directly by the duration of daylight. Even in the ferret the hypothesis scarcely seemed valid, because the initial breeding season changes are evident histologically in December and January (Allanson, 1932).

The converse experiment, keeping breeding season animals in daylight of winter duration, was also attempted by Bissonnette (1933), but the results were inconclusive. A somewhat similar experiment was therefore carried out in London, under the very stringent conditions of keeping the animals in total darkness except at the time of feeding. The results offer little support to the theory that length of daylight has any controlling influence on normal sexual periodicity.

II. *Technique.*

The ferrets were kept in ordinary wire cages in a large light-proof cupboard. The door was opened only for feeding and cleaning. The daily feeding occupied about 10 minutes and the weekly cleaning about another 20 minutes. The animals were therefore in total darkness for a minimum of 23½ hours per day. Some difficulty was experienced in securing adequate ventilation without admitting light. The arrangement finally devised consisted of having ventilators top and bottom, covered by series of vanes to exclude light. An electric fan was placed outside the top ventilator, and run so that stale air was drawn out of the top of the cupboard, and fresh air sucked in at the bottom. The fan was operated for half an hour night and morning, a time found sufficient to ensure adequate ventilation. Under these conditions the ferrets kept remarkably healthy.

III. *Experimental Results.*

Five males and five females were put in the dark on January 24, 1933. Their subsequent histories are recorded below.

Males.—At the time of going into the dark the testes of all the males were beginning to show the first signs of development, as determined by palpation. Development proceeded unchecked and a month later the testes of four animals were of practically full size. One testis of the remaining animal (DBF 2) was also well developed, but the other was still inguinal and small. This animal remained in the same condition till killed in June, being apparently a natural unilateral cryptorchid. Early in March, DBF 1, 3, 4 and 5, were in full sexual activity and remained in this condition until killed. One (DBF 4) was paired on April 29; copulation was normal and the female became pregnant. Another (DBF 5) was killed for histological examination on the same date. The remaining ones were killed on June 9.

Table I. -Male ferrets kept in the dark.

No.	Date put in dark.	Date of killing.	Days in dark.	Body weight (gm.).	Body weight at killing (gm.).	Weight of testes (gm.).	Weight of epididymides (gm.).
DBF 1	24.1.33	9.6.33	136	1320	1400	2·77	0·673
DBF 2	24.1.33	9.6.33	136	1060	920	1·07 (1 only)	0·422
DBF 3	24.1.33	9.6.33	136	1240	1120	2·55	0·566
DBF 4	24.1.33	9.6.33	136	1440	1330	3·52	0·773
DBF 5	24.1.33	29.4.33	95	1000	1050	3·93	0·730

Except for the one testis of DBF 2, the weights (Table I) were all comparable with those given by Allanson (1932) for the normal breeding season testis, and the histological appearance in each animal indicated full spermatogenic and endocrine activity, fig. 1, Plate 1. The epididymides of all were crowded with sperm, fig. 2, Plate 1.

Females.—The first sign of activity among the females was observed as early as March 10, when the vulva of DBF 10 showed signs of enlargement. A fortnight later the vulva of DBF 6 was seen to be swelling, and two other animals began in April, on the 7th and 13th. These four animals therefore began to come into œstrus within the normal range of time. It was noticeable, however, that the time taken for the vulva to become enlarged fully was longer than normal. In one animal no less than 6 weeks elapsed between the beginning and the completion of enlargement. The remaining animal (DBF 9) showed no sign of activity until May 16, and must therefore be considered as having been late in coming into œstrus. DBF 6 was killed in full œstrus on April 29. DBF 7 was mated on April 29, after which the vulva regressed in the usual way. A litter of nine was born on June 9, six of which were suckled normally until removed on July 8. By July 23 the female was in full œstrus again, at which time she mated and was killed. DBF 7 is thus of particular interest in having come into œstrus again after lactation, nearly at the end of the normal breeding season, after 5½ months of almost total darkness.

The remaining three animals were killed still in full œstrus, on June 9.

Histological examination showed that, in all the females, the œstrous condition of the vulvæ was accompanied by ripe follicles (Table II) in the ovaries and well-developed uteri, figs. 3 and 4, Plate 1.

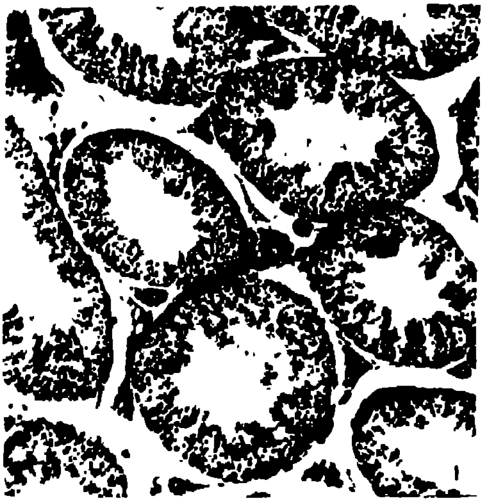
Table II.—Female ferrets kept in the dark.

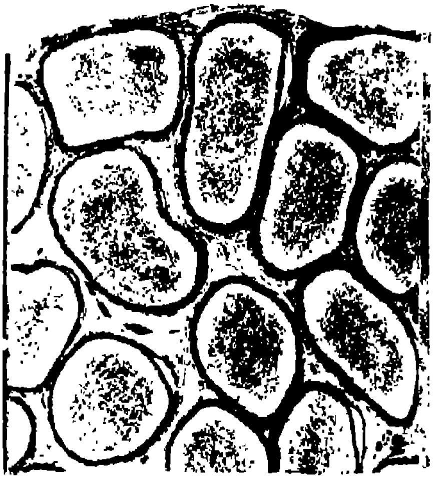
No.	Date put in dark.	Date of killing.	Days in dark.	Body weight (gm.).	Body weight at killing (gm.).	Date vulva began to enlarge.	Weight of ovaries (gm.).	Weight of uterus (gm.).	Diameter largest follicle (mm.).
DBF 6.	24.1.33	29.4.33	95	640	750	22.3.33	0·076	0·608	1·17
DBF 7.	24.1.33	23.7.33	180	800	—	7.4.33	0·109	0·952	1·34
DBF 8	24.1.33	9.6.33	136	400	520	13.4.33	0·062	0·595	1·24
DBF 9	24.1.33	9.6.33	136	420	680	16.5.33	0·063	0·794	1·32
DBF10	24.1.33	9.6.33	136	540	700	10.3.33	0·066	0·636	1·36

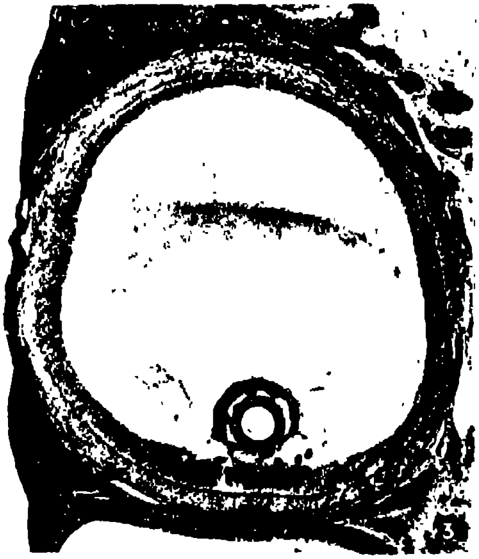
IV. *Discussion.*

The experiments recorded above make it quite evident that the almost total exclusion of light does not seriously affect the onset of the breeding season in the ferret, and therefore suggest strongly that the increasing length of daylight in the spring is not a factor influencing the normal sexual periodicity of this species. This conclusion is in accordance with the fact that the breeding season changes in the ferret can first be detected histologically in December.

Bissonnette's results, which have been amply confirmed, show quite definitely that additional illumination will induce œstrus during the winter,







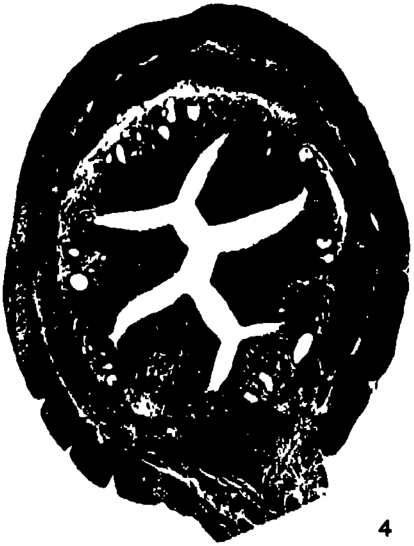


Fig. 1. Testis of DBF 5, killed April 29, after 95 days in the dark, showing normal condition. × 102.

Fig. 2. Epididymis of DBF 3, killed June 9, after 136 days in the dark showing abundance of sperm. × 58.

Fig. 3. Ovary of DBF 9, killed June 9, after 136 days in the dark, showing ripe follicle. × 50.

Fig. 4. Uterus of DBF 10, killed June 9, after 136 days in the dark, showing typical oestrous condition. × 19.

and this effect has been shown to depend on the presence of the pituitary body. (Hill and Parkes, 1933). It is, therefore, necessary to suppose that the pituitary body during anœstrus can be artificially stimulated by extra light, but that extra light is not an essential condition for the onset of the normal breeding season. A converse analogy may be seen in the fact that anœstrus can be caused experimentally by dietary deficiency, although in many animals dietary factors can play no part in the causation of normal anœstrus. It seems preferable to regard anœstrus as merely one phase of the œstrous cycle, which is known to depend on some inherent rhythm of the anterior pituitary body. In other words, the problem of the causation of anœstrus in the ferret is analogous to the problem of the causation of the five-day cycle of the unmated mouse.

Our thanks are due to Mr. J. Hammond, F.R.S., who examined the animals during the course of the experiment, and confirmed our diagnosis of their condition.

V. *Summary.*

(1) Five male and five female ferrets were kept in total darkness for 23½ hours per day from the end of January onwards.

(2) The males showed full breeding season development at the usual time and maintained the condition until killed in June.

(3) Four of the five females began to show the vulval swelling typical of œstrus by the middle of April, *i.e.*, at the usual time. The only unusual feature observed was that the swelling took rather longer than normal to reach full development. The remaining female failed to show signs of activity till the middle of May, and was therefore late.

(4) A pair of these animals were mated, and a normal litter was produced. The female subsequently came into œstrus again, towards the end of the season, after 5½ months in the dark.

(5) It is concluded that while additional light will induce œstrus in anœstrous animals, the onset of the breeding season in the spring is not dependent on the increasing length of daylight.

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Experimental Studies on the Transmission of Gapeworm (Syngamus trachea) *by Earthworms.*

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[Plate 2.]

For some time past it has been known that earthworms have a definite role in the transmission of gapeworm (*Syngamus trachea*) to birds. As early as 1886 Walker established this fact and claimed to have discovered Syngamus larvæ encysted in the gut of the earthworm. He says " . . . we generally find the embryos a short distance behind the gizzard, not often more than half-way to the tail." He was of the opinion that the earthworm was a definite intermediate host of the gapeworm. Though he cites *Lumbricus terrestris* in his title, he is less explicit in his text and we are left to assume the species when he mentions "earthworms." The fact of the transmission of gapeworm by earthworms seems to have been overlooked, except for a short note by Seurat in 1916, until Waite confirmed it in a paper, which appeared in 1920, and established the fact that it is the earthworms which carry the disease over from year to year.[*]

Although, as result of these two excellent series of experiments, there remained no doubt that earthworms are an important factor in the transmission of gapes, yet three problems still remained to be solved. They are (1) the species of earthworm concerned in the passage of the gapeworm, (2) the position in the earthworm where the larvæ may be found, and (3) the condition or state of development of the larvæ in the earthworm. The writer has conducted a series of experiments which throw some new light on all these problems. The experiments and results are described below.

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[*] [*Note added in proof, March* 9, 1933.—Since this paper was printed the writer's attention has been drawn to an article on poultry parasitosis by Cram ('North American Veterinarian.' vol. 9, p. 47, November, 1928) in which she mentions the fact of the transmission of gapeworm by earthworms. She says that she has found the larvæ encysted in the body muscles of the earthworm and that further work is being carried out. There have, however, been no publications on the subject since then.]

Material.

Two species of earthworms were obtained from ground which was unlikely to have been naturally contaminated with gapeworm eggs. *Lumbricus terrestris* was obtained from a plot of garden soil. This was watered assiduously through many weeks of dry weather until eventually the Lumbricus migrated to the surface. Large numbers of *Eisenia fœtida* were recovered from around the roots of tomato and cucumber plants in a greenhouse. The soil had been manured with artificials and so no infection could have been introduced by that means.

All these earthworms were maintained in the laboratory in the following manner: they were placed in groups of ten in large petri-dishes filled to the top with soil which had been sterilized by steaming for an hour. This soil was kept very damp. They were covered with a square of butter muslin secured with a rubber band. At the end of a week, they were transferred to fresh soil, it being estimated that all the garden soil would have been eliminated by that time, together with any free eggs or larvæ of helminths that might have been in the gut. After having been transferred to this soil, the infective eggs of Syngamus were washed into the dish and the earthworms remained in contact with this infective soil for at least 14 days before feeding to the chickens. Immediately before feeding, the earthworms were washed and brushed with a camel hair brush to remove any particles of soil and any larvæ or eggs that might be adhering to the surface.

A large number of birds infested with gapeworm was made available through the co-operation of Mr. A. D. Middleton, of the Bureau of Animal Population, Oxford University Museum. My thanks are due to him for this very considerable help.

Syngamus trachea was obtained from partridges, pheasants, chickens, and rooks and *S. merulæ,* described by Baylis in 1926, was recovered from the blackbird. Certain of the gapeworms out of each batch were always identified. This identification was considered important owing to the presence of the latter species from the blackbird which was found to be transmissible to chickens.

Syngamus trachea eggs were obtained by dissection of adults which had been removed from the tracheæ of infected birds. They were cultivated in petri-dishes on the bench in the laboratory where they reached the infective stage in about 10–14 days. They were kept free from bacterial or fungoid infections by daily aeration and agitation and frequent changes of water.

The chicks were all incubator hatched, secured as day olds and reared indoors in batteries under parasite-free conditions.

The Importance of Eisenia fœtida.

From results which will be described below it is obvious that *Eisenia fœtida* is an extremely important factor in the transmission of gapeworm disease to chickens. *Lumbricus terrestris* will also carry the disease, but infestation with gapeworm following the ingestion of *Lumbricus terrestris* is less certain and always of less severity than that following the ingestion of *Eisenia fœtida.* In this connection it is important to bear in mind one characteristic of *Eisenia fœtida.* It is always found abundantly on contaminated land and in soil containing quantities of decaying organic matter. Hence it can always be found in large numbers on land over which stock is being reared intensively such as poultry runs and pig-sties. For this reason it will be a great source of danger to young chickens which are reared year after year on old land. The presence of encysted Syngamus larvæ seems to have no effect on the vitality of the earthworm and hence they act as cumulative reservoirs of infection. There are many dangers which beset Syngamus larvæ free in the soil—in particular there is desiccation. They cannot withstand complete desiccation for many hours (Ortlepp, 1923), but in the tissues of the earthworm they have found a safe environment, where they may accumulate and mature till they reach the final host. The entry into an intermediate host always ensures greater possibilities of survival among parasites and serves as a means of overcoming the adverse conditions that are found outside.

Experiments.

The importance of *Eisenia fœtida* in the transmission of gapes was suggested by an observation made in an earlier series of experiments (Morgan and Clapham, 1934). Owing to the very dry summer of 1933, earthworms were scarce and *Eisenia fœtida* was the only species obtained in any quantity. As excellent results were always obtained with it, it was decided to follow up the results.

Eisenia fœtida and *Lumbricus terrestris* were infected with *Syngamus trachea* from various sources and were then fed to chickens. The feedings were conducted in the following manner. The chicks were divided into five groups :—

Group A fed with infected *Lumbricus terrestris.*
Group B fed with uninfected *Lumbricus terrestris.*

Group C fed with infected *Eisenia fœtida.*

Group D fed with uninfected *Eisenia fœtida.*

Group E fed with infective eggs of *Syngamus trachea* direct. They were the controls.

In all experiments chickens in groups B and D remained completely uninfected, showing that the earthworms contained no natural infection. In group C (fed artificially infected *Eisenia fœtida*) 100% infections with gapeworm were the result. Disease was produced from gapeworms obtained from the partridge, pheasant, rook, and chicken. Usually the chicks died and post-mortem examination of the trachea showed it to be completely blocked with gapeworms. In many cases the gapeworms were so numerous that the bird died before the gapes could mature. The number present varied from 6 to 51 pairs. About 50% of the chickens which were fed infected *Lumbricus terrestris* (group A) became infested with the gapeworm, but the infestations were never heavy enough to cause death. The numbers of pairs of gapeworms resulting from these infections never exceeded 8. In group E, fed 200 infective eggs of *S. trachea* direct, less than 25% of the chickens became positive. Usually the number of gapeworms recovered was either 1 or 2, but on one occasion 5 pairs were obtained.

Investigations are now being made as to the importance of *Eisenia fœtida* in the transmission of *Syngamus trachea* from starlings, this is said to be a distinct strain and not easily transmissible to chickens.

The details of these experiments are shown in Tables I–IV.

Using *Eisenia fœtida*, the writer was able to transmit *Syngamus merulæ* from blackbirds successfully to chickens, thus showing that this species may also be a source of danger to poultry. (Table V.)

From these experimental results we can obtain the answer to our first problem—that of the species involved in the transmission of gapes. *Eisenia fœtida* is certainly very important in this respect. *Lumbricus terrestris*, though a potential intermediate host, is less important.

Our other two problems were solved by dissection of the earthworm.

Examination of the Artificially Infected Earthworm for Larvæ of Syngamus trachea.

Specimens of *Eisenia fœtida* which had been artificially infected with larvæ of *Syngamus trachea* were killed by drowning. A median longitudinal incision was made dorsally and the gut lifted out whole. The gut and body wall were

Table I.—Experiment using *Syngamus trachea* from the Partridge.

Material fed.	No. of chicks used.	Age of chicks in days.	Numbers.	Dose.	No. of positive infections.	No. of pairs of gapes at autopsy.
Group A. Infected *L. terrestris*	8	4	2–9	3 earthworms	4	No. 2—8 „ 3—3 „ 4—6 „ 5—0 „ 6—0 „ 7—4 „ 8—0 „ 9—0
Group B. Uninfected *L. terrestris*	8	4	10–17	3 earthworms	0	0
Group C. Infected *E. fœtida*	8	4	18–25	5 earthworms	8	No. 18 51 „ 19—31 „ 20—16 „ 21 - 28 „ 22—10 „ 23 32 „ 24—25 „ 25—19
Group D. Uninfected *E. fœtida*	8	4	26–33	5 earthworms	0	0
Group E. Infective eggs in water	8	4	34–41	200 eggs of *Syngamus trachea*	2	No. 34 0 „ 35—0 „ 36—0 „ 37—2 „ 38 -0 „ 39—5 „ 40—0 „ 41—0

All the chicks died in Group C.

cut into small portions of a convenient size and examined by press preparations in Ringer's solution. The gut was examined with particular care as Walker claimed to have found Syngamus larvæ behind the gizzard. The present writer was, however, unable to find any gapeworm larvæ in any portion of the gut. Larvæ were, however, obtained from the body wall. Fig. 2, A, Plate 2, shows the larva encysted in the body wall and fig. 2, B, after removal. The bulk of them were found in the middle region of the earthworm, but specimens were recovered from along the whole length both dorsally and ventrally. They occurred deeply embedded in the muscles, in a thin hyaline cyst. When they

Table II.—Experiment using *Syngamus trachea* from Pheasants.

Material fed.	No. of chicks used.	Age of chicks in days.	Numbers.	Dose.	No. of positive infections.	No. of pairs of gapes at autopsy.
GROUP A. Infected *L. terrestris*	7	5	4–10	3 earthworms	4	No. 4—0 „ 5—6 „ 6—1 „ 7—0 „ 8—0 „ 9—3 „ 10—0
GROUP B. Uninfected *L. terrestris*	7	5	11–17	3 earthworms	0	0
GROUP C. Infected *E. fœtida*	7	5	18–24	4 earthworms	7	No. 18—11 „ 19—16 „ 20—25 „ 21—6 „ 22—13 „ 23—28 „ 24—26
GROUP D. Uninfected *E. fœtida*	7	5	25–31	5 earthworms	0	0
GROUP E. Infective eggs in water	6	5	32–37	200 infective eggs of *S. trachea*	1	No. 32—0 „ 33—1 „ 34—0 „ 35—0 „ 36—0 „ 37—0

Nos. 20, 23, 24 died of the disease. All the others in Group C gaped badly.

were dissected out from these cysts in Ringer's solution, they showed considerable activity. From their anatomical structure, they could be recognized as third stage larvæ of *Syngamus trachea.* The characteristic feature is the shape of the buccal rods, a drawing of which is given, fig. 1, as previously no adequate representation of them has appeared. They had not developed appreciably from the stage seen in the infective egg. They were, however, ex-sheathed. As, however, other nematodes are known to encyst in earthworms, one of which, *Rhabditis pellio,* closely resembles *Syngamus trachea,* this morphological similarity was not considered sufficient for identification. Therefore 53 cysts, believed to be Syngamus larvæ, were isolated and fed to a chicken: 17 days later the chick died and showed 16 pairs of gapeworm in the trachea. This is definite proof of the identity of these larvæ.

Other specimens of *Eisenia fœtida* which had not been infected with Syngamus larvæ were also dissected, but no structures resembling these cysts were found in the infected earthworms. We have therefore three proofs of the identity of these cysts, (*a*) their absence in non-infected earthworms, (*b*) the structure of the buccal rods of the contained embryos, and (*c*) the production of gapeworm after feeding to chickens.

Table III.—Experiment using *Syngamus trachea* from Rooks.

Material fed.	No. of chicks used.	Age of chicks in days.	Numbers.	Dose.	No. of positive infections.	No. of pairs of gapes at autopsy.
Group A. Infected *L. terrestris*	4	13	3–6	2 earthworms	2	No. 3—0 ,, 4—2 ,, 5—4 ,, 6—0
Group B. Uninfected *L. terrestris*	4	13	7–10	2 earthworms	0	0
Group C. Infected *E. fœtida*	4	13	11–14	4 earthworms	4	No. 11—18 ,, 12—7 ,, 13—2 ,, 3—3
Group D. Uninfected *E. fœtida*	4	13	15–18	4 earthworms	0	0
Group E. Infective eggs in water	4	13	19–22	200 eggs of *S. trachea*	1	No. 19—0 ,, 20—1 ,, 21—0 22—0

Other Nematodes Encysted in Earthworms.

Considerable care was necessary when dissecting these earthworms as several species of nematodes are known to encyst in the cœlom and muscles of various earthworms. Species of at least six genera have been recovered; they are *Drilonema vendessianum* (Pierantoni, 1916), *Pierantonia microcephalum* (Pierantoni, 1916), *Pharyngonema mekongianus* (Pierantoni, 1923), *Dicelis filaria* (Wülker, 1926), *Ungella secta* (Cobb, 1928), and *Rhabditis pellio* (Johnson, 1913; Otter, 1933). Of these, the first five are unimportant in this series of observations. Most of them are rare, occur as adults and all are easily recognizable. They could not be confused with *Syngamus trachea*.

Table IV.—Experiment using *Syngamus trachea* from Chickens.

Material fed.	No. of chicks used.	Age of chicks in days.	Numbers.	Dose.	No. of positive infections.	No. of pairs of gapes at autopsy.
GROUP A. Infected *L. terrestris*	3	11	1-3	2 earthworms	1	No. 1—2 ,, 2—0 ,, 3—0
GROUP B. Uninfected *L. terrestris*	3	11	4-6	2 earthworms	0	0
GROUP C. Infected *E. fœtida*	3	11	7-9	3 earthworms	3	No. 7—4 ,, 8—10 ,, 9—8
GROUP D. Uninfected *E. fœtida*	3	11	10-12	3 earthworms	0	0
GROUP E. Infective eggs in water	3	11	13-15	200 infective eggs of *S. trachea*	1	No. 13—0 ,, 14—1 ,, 15—0

Table V.—Experiment with *S. merulæ* from the Blackbird.

Material fed.	No. of chicks used.	Age of chicks in days.	Numbers.	Dose	No. of positive infections.	No. of pairs of gapes at autopsy.
GROUP C. Infected *E. fœtida*	3	7	3-5	1 earthworm	2	No. 3—0 ,, 4—2 ,, 5—1
GROUP D. Uninfected *E. fœtida*	3	7	6-8	1 earthworm	0	0
GROUP E. Infective eggs in water	5	7	9-13	100 eggs of *S. merulæ*	0	0

Drilonema can be distinguished by its short œsophagus and blunt tail of relatively great length. Furthermore, it occurs free in the cœlom of the earthworm. *Pierantonia* is characterized by the fact that the œsophagus is non-muscular and is not distinct from the intestine. The two sexes are found *in copula*. This also occurs in the cœlomic cavity. *Pharyngonema*, which is another genus found free in the cœlom, is distinguished by its swollen neck region while *Dioelis* only occurs in the sperm sacs. In any case it carries a lateral sucker on the tail quite unlike any other nematode. *Ungella*, which closely resembles and may indeed be identical with *Syphacnema* (Baylis and Daubney, 1926), may occur in the muscles, but this would be easily recognized by the mouth cavity, which is overhung by a pair of curious curved hooks.

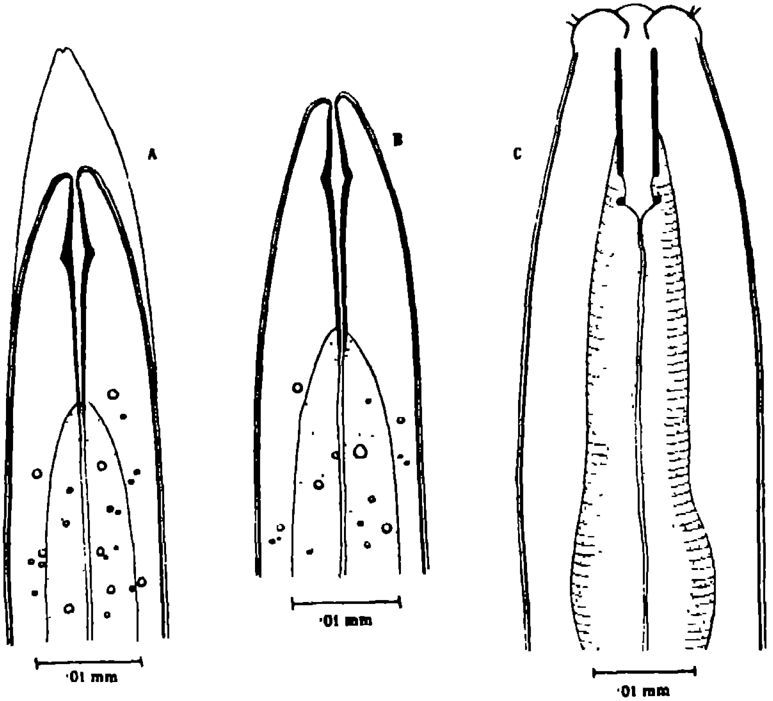

FIG. 1.—A and B : Third stage larvæ of *Syngamus trachea*. Anterior end showing characteristic buccal rods. A : Infective larva, just hatched from egg. The larva is sheathed. B : Infective larva, dissected from muscles of earthworm, *Eisenia fœtida*. The larva is ex-sheathed. C : Larva of *Rhabditis pellio*, recovered from the earthworm. Anterior end showing typical rhabditiform buccal rods.

Rhabditis pellio, however, occurs as a larva in the earthworm where it often encysts among the muscles. It bears some resemblance to the Syngamus larva and high magnification is necessary before identification can be made with certainty. It, however, usually congregates at the posterior end of the body and encysts in well-developed brown cysts, quite distinct in appearance from the thin transparent ones of the Syngamus larva. Two other differences occur internally in the larva itself. In the second œsophageal bulb in *R. pellio* there is present the cuticular masticatory apparatus characteristic of the genus *Rhabditis*. This is absent in the Syngamus larva. Then, too, the buccal rods are of the typical Rhabditis shape and appearance and quite different from those in Syngamus. Free specimens are often found when seeking Syngamus larvæ, but they can readily be distinguished, not only by the morphological characters, but also by the fact that they are very active. In *Eisenia fœtida*, however, *Rhabditis pellio* is rare (Otter, 1933).

Infection of Older Chickens with Syngamus trachea.

It is generally stated in the literature that infection with *Syngamus trachea* can only be established in very young chickens. They are susceptible to the worm after hatching, but they gradually develop a resistance which is complete by the time they are 6 weeks old. But there have appeared from time to time accounts of older chickens harbouring one or more pairs of worms. Waite (1930) believes that he transmitted gapeworms to yearling hens using a large number (150) of infected earthworms. Fifteen days later, two out of three hens had gapeworm. He says, " worms could be distinctly seen in the trachea of two of them by looking down their throats in good light. Two weeks later these worms had disappeared. The hens were killed July 20 and examined, but no signs of gapeworms were found." It would have been more conclusive if he had post-mortemed the hens when he believed they were harbouring the Syngamus or at any rate examined the droppings for eggs.

Morgan, however (1931), records an undoubted case of gapeworm infection in a yearling hen. At post-mortem examination 12 pairs of gapeworms were found in the lower half of the trachea.

Old chickens rendered susceptible by means of dietary deficiency.

It is, however, extremely rare to find old chickens infected and very difficult to bring about experimental infections, and the following experiment is of interest :—

Twelve chickens were used. At the age of 10 weeks they were divided into three groups and fed as follows :—

Group A was fed a diet in which vitamin A was completely lacking. It consisted of a balanced mixture of steamed casein, tapioca meal, white maize, salt, limestone grit, oyster shell, and olive oil.

Group B was fed a diet very low in minerals, particularly calcium. This consisted of yellow maize, white maize, tapioca meal, cod liver oil, salt, and cooked lean meat.

Group C was fed a normal nourishing diet, adequate in all respects.

After being on these experimental diets for 3 weeks, groups A and B showed obvious signs of deficiency. All the chicks were then given four *Eisenia fœtida* which had been infected with *Syngamus trachea.* After 15 days one bird in group B showed the sniffle and twitching of the head characteristic of gapeworm disease. Fæces examinations were made and in groups A and B Syngamus eggs were demonstrated. All the birds were therefore killed. Post-mortem examination showed that three birds in both groups A and B were infected with *Syngamus trachea.* In each group one bird was free. No gapeworms were found in the controls, group C.

Fig. 3, Plate 2, is a photograph of the infected tracheæ and also three of the control tracheæ which, however, show only the normal healthy appearance.

The numbers of gapeworms recovered were: Group A, 3, 6, 2; group B, 4, 1, 2; group C, 0.

A similar experiment had been carried out some months ago before the writer realized the importance of *Eisenia fœtida* in the transmission of gapeworm. She was able to infect one three-months-old cockerel on vitamin A deficient diet with a single pair of gapeworms using the direct method of feeding with infective Syngamus eggs.

It would seem from these results, that using *Eisenia fœtida* as intermediate host, it is possible to induce infection with gapeworms in older chickens which are undernourished.

Acknowledgments.

These experiments have been carried out in the laboratories of the Institute of Agricultural Parasitology, St. Albans, under the direction of Professor R. T. Leiper, M.D., D.Sc., F.R.S., to whom I am much indebted for his unfailing assistance, by suggestion and by criticism, throughout the course of the work.

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I am indebted to Mr. W. A. McDonald, of the London School of Hygiene and Tropical Medicine, for taking the photographs.

Summary.

(1) *Eisenia fœtida,* an earthworm commonly found in contaminated soil, is shown to be the important intermediate host of *Syngamus trachea,* the common gapeworm of birds.

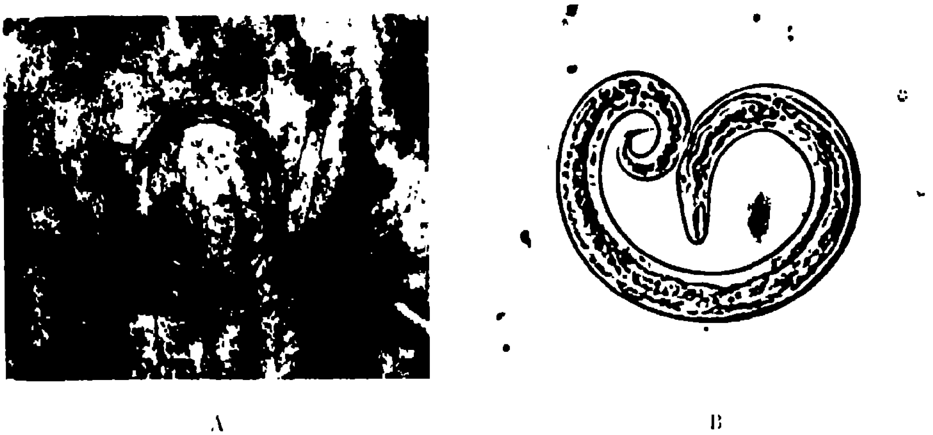

FIG. 2. Photographs of third stage larva of *Syngamus trachea*, from the earthworm, *Eisenia fœtida*. A, larva encysted in muscles of body wall × 250. B, larva dissected from muscles, × 370.

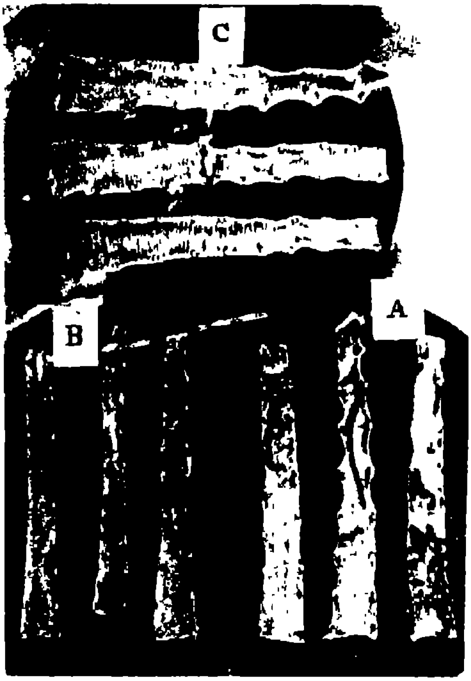

FIG. 3. A, tracheæ of three old chickens, fed a diet deficient in vitamin A, experimentally infected with *Syngamus trachea*. B, tracheæ of three old chickens, fed a diet deficient in minerals, experimentally infected with *Syngamus trachea*. C, tracheæ of control chickens fed a normal diet. They were immune to infection with *Syngamus trachea*.

(2) *Lumbricus terrestris* also acts as intermediate host but is less important.

(3) Using *Eisenia fœtida*, chickens have been infected with *Syngamus trachea* originating from pheasants, partridges, rooks, and chickens.

(4) *Syngamus merulæ* also has been transmitted to chickens via *Eisenia fœtida.*

(5) The third stage larva of *Syngamus trachea* encysts in the muscles of the body wall of these earthworms and may remain dormant there until taken in by a chicken or other suitable host, when they hatch and may develop into gapeworms.

(6) No further development of the Syngamus larva occurs in the earthworm. They remain as third stage larvæ, but they have lost their sheaths.

(7) They are unlikely to be confused with any other nematodes which have been found in the earthworm, except *Rhabditis pellio.*

(8) The larvæ of *Syngamus trachea* in the earthworm can be differentiated from those of *Rhabditis pellio* by the characteristic shaped buccal rods and the absence of any cuticular valved apparatus in the œsophageal bulb.

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Metachronal Rhythms and Gill Movements of the Nymph of Caenis horaria *(Ephemeroptera) in Relation to Water Flow.*

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Introduction.

The gills of some Ephemerid nymphs are always motionless, *e.g.*, many Bætine forms of English streams. In many others, however, the gills move rapidly in metachronal rhythm, by virtue of which currents are created in the water. These currents are peculiar to the species and probably have an adaptational significance. In many forms already under investigation, *e.g.*, *Chloen dipterum*, *Siphlurus* sp. *Ecdyonurus venosus*, *Ephemerella* sp. *Leptophlebia marginata* and *Ephemera vulgata*, a common feature is noticeable. This is that in their rhythmical movements both members of each pair of gills beat together, *i.e.*, and their movements are co-phasedly synchronized. Since, therefore, the effect of the gills on one side of the body is exactly duplicated on the other, whatever may be the precise mechanism for the production of currents, the latter are symmetrical with the longitudinal axis of the body (Eastham, 1932).

An interesting exception is the nymph of *Caenis horaria*. In this animal the currents pass over the body from one side to the other. The gills beat in metachronal rhythm down each side of the body, but though the rhythms are synchronous there is a time phase difference between them. In other words, members of a pair are not co-phasedly synchronized in movement. We have thus in *Caenis horaria* a bi-laterally symmetrical animal producing movements in the surrounding medium which are not of the nature of an axial flow. It is with this phenomenon that this paper deals.

Methods.

The currents produced in water by the movement of the gills are easily determined by observing fine particles in suspension. Suspensions of fine mud serve quite well for this purpose as also do cultures of Ciliate Protozoa. The latter have an advantage over non-living suspensions since they do not come to rest. Ordinary microscopic methods served for observing currents. Metachronal movements of the gills in relation to the latter were examined by means of a stroboscope. This instrument, fashioned on the lines described by Gray (1930), consists of a perforated disc rotated by an electric motor the speed of which is regulated by a variable resistance. An electric light is mounted behind the disc and the whole is enclosed in a wooden box firmly screwed to the bench.

The gills lie flat over the back of the animal so making observation in dorsal view by transmitted light almost impossible. Direct illumination from the stroboscope in a dark room gave better results, though still more satisfactory views were obtained by observing the animals in side view by transmitted light. For this purpose the nymphs were fixed on their sides between small glass plates. Fine entomological pins, inserted through the wing covers and bent at appropriate angles served well for holding the nymphs under observation in different attitudes. In this way a clear picture was obtained of all aspects of the gills in slow motion. The gills are easily detached from the body and thus various combinations of gills in action could be observed after removal of the others. Comparison was then possible with the effects produced by the complete set of gills. To avoid the possible ill-effects associated with mutilation a fine needle was also employed to hold certain gills motionless while observations were made on the remainder which were free to move.

The frequency of gill oscillation was measured by means of a speedometer fixed to the motor spindle. The speed of the stroboscope disc (the frequency of illumination) was then adjusted until the gills appeared to be motionless, frequency of oscillation and of illumination being then identical.

To observe the gills in slow motion the motor was speeded up to a rate beyond that of the oscillating gills. It was then gradually slowed down until the gills appeared to be perfectly still. A further slight reduction in the frequency of illumination then gave a clear slow-motion picture of the gills, their apparent direction of motion under these conditions being the same as their actual motion.

With the stroboscope operating under these conditions it often happens that the frequency of oscillation falls below the light frequency. The gills then appear to be rising when they are actually falling and *vice versa.* Unless this is recognized the observer runs the risk of jumping to strange conclusions as to the mechanism involved in current production. Every care was taken in this work to avoid the pitfalls coincident with the phenomenon of stroboscopic reversal (Gray, 1930).

The Gills.

The nymph of *Caenis* possesses six pairs of gills on the first six segments of the abdomen. The first pair are slender, immobile, tactile processes attached to the postero-lateral angles of the first segment. The second pair similarly attached to the next segment have the form of sub-quadrangular plates resembling the elytra of certain adult insects. I shall refer to these as the pseudo-elytra. When at rest they overlap slightly in the middle line of the body and extend posteriorly so as to cover the gills of the next succeeding four abdominal segments. They can be raised to a stationary position at an angle of about 30° to 40° with the body surface while the remaining four pairs of gills oscillate beneath them. The pseudo-elytra never oscillate, and doubtless their main function is protective and to a lesser extent respiratory.

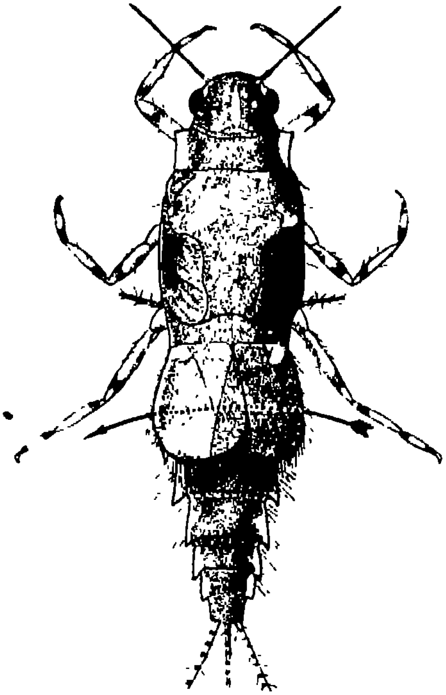

Fig. 1.—The nymph of *Caenis horaria* to show the current passing under the pseudo-elytra from one side of the body to the other. The first pair of gills are represented as a setose process projecting at each side.

The four pairs of gills behind them, which in this paper will be referred to as Nos. 1, 2, 3, and 4, are similar to each other except in size, fig. 2. Each consists of a somewhat semicircular plate slightly concave below and convex above, and articulated to the posterior angle of its segment by a narrow stalk. The gill, fig. 3, when at rest lies nearly flat on the body with its straighter border towards the front. Each overlaps the next one behind it. Passing through the gill-plate is a rich system of branching tracheæ, the fine ends of which pass into numerous fine filaments with which the gill is fringed. The distal marginal filaments on the gill are longest. The proximal ones are short and a small part of the proximal anterior border is fringeless. Except for the shortest proximal ones the marginal filaments are branched—the longer the filament the more branched is it. Such branches invariably pass ventrally to neighbouring main filaments in regular arrangement as shown in fig. 2.

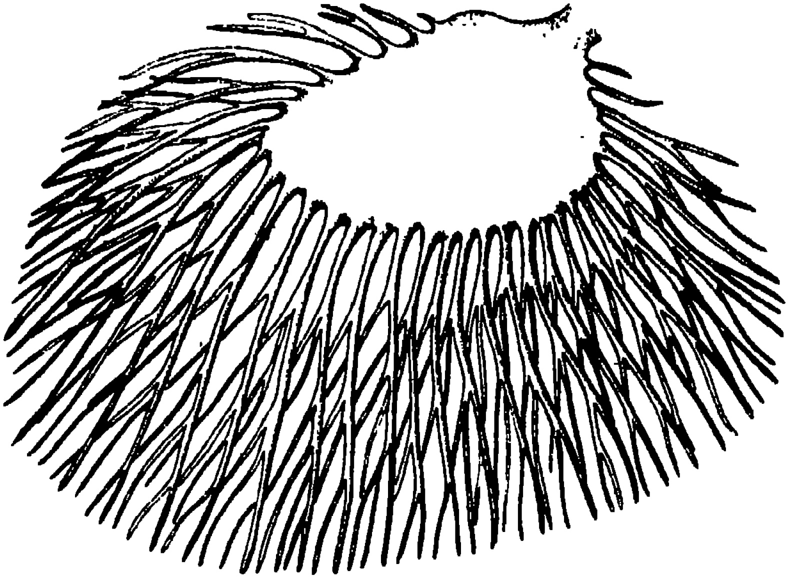

FIG. 2.—Dorsal view of the 1st oscillating gill (morphological 3rd) of the right side. Note the branches of the marginal filaments.

The members of any pair of gills are normally equal in size, but any gill is greater than the one next behind it. This difference of size is of such an order that the posterior marginal filaments of one gill extend nearly as far back as do those of the one next behind it. In the resting position the distal borders of a pair of gills nearly meet in the middle line, the marginal fringe of one overlapping that of the other. Each gill of the right side overlaps its fellow of the other at a particular moment. At other times the reverse condition applies. An individual can and does change the arrangement of its gills in this respect.

Water Currents caused by the Gills.

The main current is observed to enter the space beneath the pseudo-elytra at one side and pass out at the other, fig. 1. The direction of this lateral flow may be from either side to the other. If the pseudo-elytra be removed no appreciable difference in the current is observed, either in direction or strength. Small eddies from the sides of the thorax pass backwards and join the main transverse flow. A current, so weak as at most times to escape detection, passes from before backwards along the distal edges of the gills. This probably occurs when the pseudo-elytra are present, but owing to the opacity of these organs this cannot be determined. It may, I think, be safely assumed that the pseudo-elytra are not concerned with any directional effect on the current.

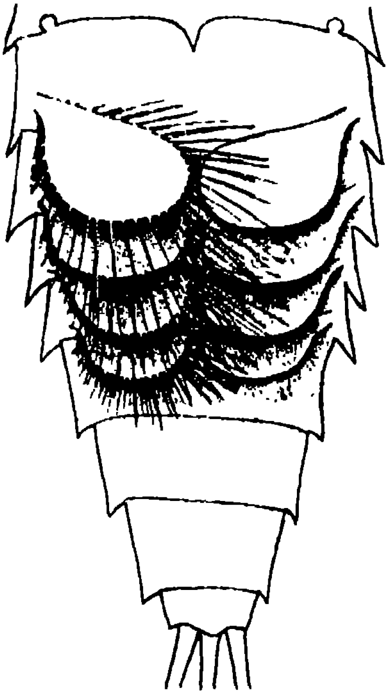

FIG. 3.—View of four pairs of gills at rest after removal of the pseudo-elytra. The filaments of those of the left side only are shown in very simplified form.

The flow of water is not continuous but pulsating, the pulsations coinciding with the gill oscillations. In this pulsating flow each thrust in the main direction is preceded by a shorter and less pronounced movement in the opposite direction. An individual can reverse its current. Reversal of flow is always preceded by a momentary stoppage during which the gills are rearranged in a manner to which I shall refer later.

From time to time the nymphs of *Caenis* swim by means of slow undulations of the abdomen. During progress the gills are commonly in motion. The effect of the oscillating gills on the water is the same as when the animal is stationary. Instead, however, of moving the water over its body the body is made to roll over sideways in a direction opposite to that in which the water is made to move. Thus in addition to the forward propulsion of the animal produced by abdominal undulations there is a body spin imparted by the oscillating gills. That this is due to the gills can be readily shown by their removal, when the animal swims dorsal side up. The spin imparted to the body described above is of some advantage to the animal. As *Caenis* swims upwards it finds itself with its feet uppermost and within reach of the surface film. To the latter it clings with its claws and hanging therefrom, it proceeds for some time to pass the clear surface water over its gills. Thus by means of a strange acrobatic feat the animal can at any time and for an indefinite period explore the more oxygenated water of its environment.

Gill Movement.

When the pseudo-elytra are raised, the four pairs of gills behind and beneath them begin to oscillate. There is some slight variation in the speed of movement. Readings taken on a number of individuals gave an average of 8·5 oscillations per second. At times the rate falls to about 7 per second and in individuals becoming exhausted through removal of some of their gills it may fall so low as to make stroboscopic vision impossible (Gray, 1930). Removal of the pseudo-elytra is necessary before the gill movement can be properly seen. From the resting position each gill is raised on its stalk to an angle of about 45° with the body surface. In rising, the marginal filaments lag behind the gill plate so increasing the concavity of the undersurface. At the same time the branches of the longer filaments lag behind the latter so as to lie directly beneath and in line with them, fig. 4. On the return stroke a distinct thrust is noticeable. The flexed filaments straighten out and their branches return to their normal position, *i.e.*, so as to cross obliquely under the main filaments adjacent to them, fig. 4.

In addition to the rise and fall in periodic motion as here described, each gill pivots on its stalk in both up and down movements. The pivoting causes (i) the gill to face to one side in the upstroke and to the other in the downstroke ; (ii) the gill to traverse an elliptical path in its upward and downward movement, figs. 6 and 9.

Thus with flow from right to left the underside of each gill turns to the right in its upward beat and to the left in its downward beat. With such a current each gill passes upwards to the right-hand side of its vertical axis of motion and downwards to the left-hand side of it, fig. 6A.

With a current in the opposite direction the underside of the gill faces left on its upward and right on its downward beat. On rising, each gill moves in a half ellipse to the left of its vertical axis of motion and in falling completes its elliptical path by passing to the right of this axis, figs. 5 and 6B. That these phenomena have an important effect in determining the direction of flow will easily be realized.

Metachronal rhythm in the movement of the gills is evident as soon as the pseudo-elytra are removed. Only by means of the stroboscope, however, can such details as the phase difference between adjacent gills be determined. Since the gills overlap each other from before backwards, a view from the side or from the middle line of the body is most convenient for seeing the rhythm along a longitudinal series of gills. The phase difference between two adjacent gills is then seen to be one-third of a complete oscillation. Further, any gill has a leading phase relation to the one next behind it. Thus the first gill performs one-third of a complete oscillation, *i.e.*, passes through two-thirds of its upward stroke before the second gill of its own side begins its upward stroke. It follows from this that the rhythm is from before backwards and that the first and fourth gills of the same side are in the same phase of movement. The latter fact can be easily verified by watching the first and fourth gills after Nos. 2 and 3 have been removed. The above order of phase difference

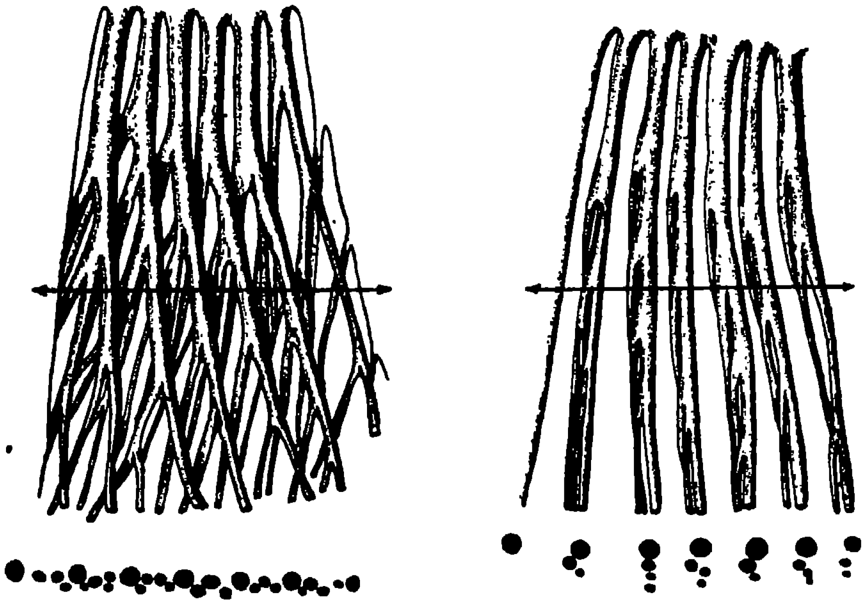

Fig. 4.—Dorsal view of a portion of the marginal fringe of gill shown in fig. 1. A, in the position assumed during the effective downstroke; B, in the upstroke attitude. Beneath each figure is shown the arrangement of filaments as seen in transverse section across the fringe along the line drawn across.

between the gills in motion is not absolute. Certain individuals had a rhythm in which the phase difference between gills in motion was such as to put the first and fourth out of phase with each other. Such variations are, however, of little moment. No form was found to have any but an antero-posterior rhythm. It is worthy of note that in all Arthropods examined by Cannon (1928), the rhythm of their limb movement was in the opposite direction.

The above facts were obtained first by using animals with all the gills intact, and later they were verified on animals from which the gills of one side had been removed. By this means a single row of gills could be observed from both lateral and median aspects of the body.

Though the gill movements are synchronous throughout, there is clearly a time phase difference between the rhythms passing along the two lateral rows of gills. The members of pairs are not co-phasedly synchronized in movement. Between members of pairs the phase difference is one-third of a complete oscillation, *i.e.*, a difference of the same order as that which exists between adjacent gills in a longitudinal series, fig. 5.

In addition, therefore, to the metachronal rhythm along the gills down each side of the body there is a second rhythm passing transversely over each of the four gill-bearing segments involving in each segment two elements, the members of a pair.

The transverse rhythm is reversible. At one time the left gill of each pair may have a leading phase relation to its fellow of the opposite side. In such animals it is found that each left gill underlies by its filaments the right gill of its own segment in the middle line of the body. Such an arrangement is invariably accompanied by a flow from the left side of the body to the right, fig. 5. At another time the right gill of each pair underlies, and has a leading phase relation to, its fellow of the left side. With this arrangement the current produced passes from right to left. It has been observed that the gills come to rest momentarily at intervals of from 30 to 60 seconds and a change in direction of flow often follows. Reversal in the transverse rhythm as described above does not involve a change in the axial rhythms themselves. The relation of one axial rhythm to the other alone is changed.

Since it is easy with the stroboscope to obtain an impression of *apparent* reversal of rhythm which is not *real*, it ought to be pointed out that every precaution was taken to avoid this error. When a view of the gills " in reverse " is obtained one sees a gill appearing to move down while in the attitude actually assumed by the gill in its upstroke. It has been pointed out that on the actual upstroke the filaments lag behind the gill plate whereas on the downstroke they straighten out. If through a too high frequency of illumination the phenomenon of stroboscopic reversal is obtained, the gills will continue to rise and fall in periodic motion. On the *apparent* upstroke, however, the

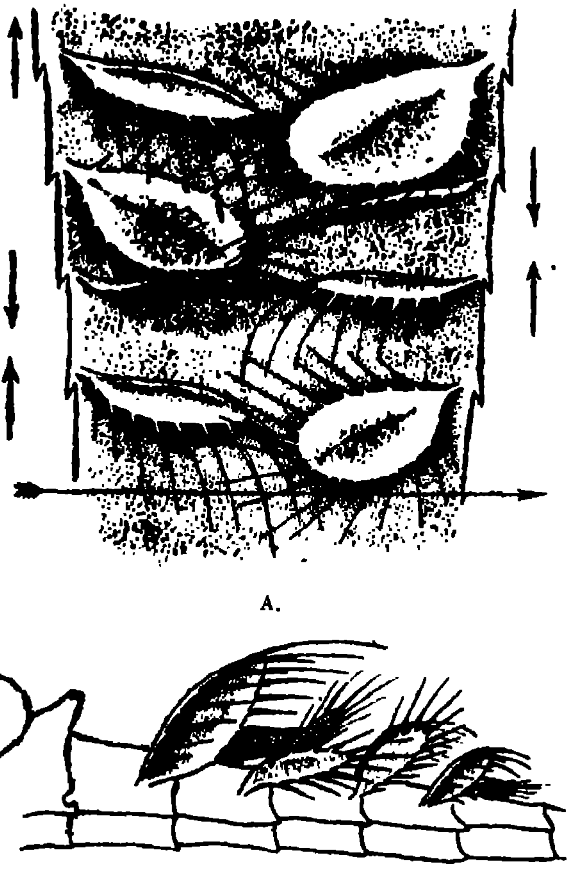

Fig. 5.—A, simplified diagram of the gills in motion as seen from above when producing a current from left to right. The arrows at the sides indicate the particular movements being performed by the gills next to them. Note that right and left series are out of phase with each other and that each right-hand gill overlaps its fellow of the left side by means of its filaments. B, diagram of a lateral view of the gills shown in fig. A. In producing the flow from left to right each gill turns its lower surface to the left in rising (see left gills 1 and 4) and to the right in falling (see left gill 3).

filaments will be outstretched and on the *apparent* downstroke they will be flexed downwards. Also under such conditions, since the gills appear to be moving up when they are actually moving down the phase relations between adjacent gills will *appear* to be reversed. Gill movement is under nervous control and the rate of oscillation may change while observations are being made. Thus if the frequency of illumination has been fixed to give a slow motion picture of actual movement, a slight lowering in the rate of gill oscillation may produce a picture of the gills in stroboscopically reversed movement and with reversed rhythm. A lower frequency of illumination will remedy this, provided that the effect of "flicker" does not necessitate too great a reduction in the intensity of light (Gray, 1930).

The Form of the Gill in Relation to Flow.

Before considering the phenomena of metachronal rhythm in relation to flow let us first discuss the possible mechanism whereby the gills cause their local flow.

An important characteristic of the gill is its marginal fringe. This I consider forms a bounding surface liable to prevent wasteful eddies. It is well known that a wall, topped with stiff grass, will, to a person sitting behind it, afford more shelter than one without such a fringe. This is because the grass can sway with the stream line of the wind, and the eddies which would otherwise be set up on the leeward side of the wall are largely diminished.' The gill fringe will act similarly in preventing eddies round the gill border. That it does so may be inferred from the weakness of the axial current which passes backwards along the gill edges (p. 34).

A second function may be attributed to the gill fringe—a function depending on the difference in its behaviour in up and downstrokes. The marginal fringe is so thick and the spaces between them so small as to offer considerable resistance to water flow between them. When the filament branches lie across their neighbours a close meshwork is formed round the gill plate which is probably for all ordinary purposes impervious to water. It is when the gill is in this position, fig. 4A, that it makes its effective beat downwards, thrusting the water from the inter-gill space between the falling gill and the one next behind it. On the upstroke, however. the filaments become flexed and their branches lagging behind, take up a position beneath and in line with the main filaments to which they belong, fig. 4B. Thus in the upstroke when the inter-gill space (between the rising gill and the one next behind it) is filling with water, there may be expected an inflow between the filaments from above. Fig. 4 represents diagrammatically surface and sectional views of the filamentar region in up and downstrokes.

Each gill therefore has a form which, like the feathers on a bird's wing, makes an impervious vane on the effective beat and a pervious vane on recovery. The different resistance of water offered to the gill in the two halves of a complete oscillation is accentuated by the curvature of the gill plate itself. In rising the upper convex surface will allow water to slide over it easily as air does over the bird's wing in its upstroke. On falling, however, the concave surface meets the water and the maximum resistance is encountered. This is the effective stroke thrusting the water away.

In connection with the above, the following measurements are of some interest. The distances between neighbouring filaments of a gill under the conditions of upward and downward movement were measured. The spaces between adjacent filaments during the upstroke vary from 0·013 mm. proximally to 0·03 mm. distally. The distance apart of the filament branches forming the meshwork during the downstroke is about 0·007 mm.

Gill Movement in Relation to Flow.

Since both axial and transverse rhythms exist the effects of both should be apparent, yet the transverse flow predominates. Even when the gills of one side only are present the flow is still to the side. We are justified, therefore, in concluding that whatever be the causes of transverse flow, the transverse rhythm is not the only factor. An analysis of the action of a single gill is obviously called for.

It was at first thought that the clue to the situation lay in the fact that the gill possessed a fringe of filaments of varying length along its border. The parts where the fringe is longest might be regarded as places where the viscous resistance to water flow is greater than the places where the fringe is shorter. On this theory a flow with a unidirectional component from the middle line of the body (longest fringe region) to the side (short fringe region) might be expected. To test this idea the delicate operation of cutting away the fringe from all the gills was performed and no difference in direction of flow was noticed. The flow was, of course, weaker, the effective area of each gill having been reduced by the operation. It follows from this that the fringe is not responsible in any marked fashion for the side flow in the manner suggested.

I have already referred to the fact that in addition to periodic up and down motion the gill executes a pivoting movement. Let us consider the gill as a flat plate oscillating in the manner described, and in relation to a lateral flow caused thereby. In fig. 6 the line XY represents the axis of water flow in the direction of the arrows, and the dotted ellipse, the path traversed by the gill. The attitudes of the gill in different parts of its path are indicated by the firm lines.

It is clear that the gill is moving at an angle with its own path of motion. Further, this angle varies as the gill performs a complete oscillation. θ is the angle between the gill surface and the vertical axis of motion, this being at right angles to the axis of flow. This angle is least when the gill is crossing the axis of flow and greatest when it is farthest away from it.

Such a set of circumstances are comparable with those described in the movements of fish by Gray (1933, *a*, *b*, and *c*). The gill of *Caenis* is related in its vertical movement to the axis of flow (across the body) in the same way as the tail of a fish is related to its own longitudinal axis of motion. Gray has shown that the net effect of moving a segment of the fish's body through the water at an angle to its own direction of motion is to impress on the body two forces, tangential and normal, the resultant of which represents the net propulsive thrust which drives the body against the resistance of the water.

With *Caenis* the animal is stationary and the forces generated are expended in producing a flow of water across the body. According to Gray, as the angle θ decreases, the thrust effected on the water increases and *vice versa*. In other words, the greatest thrust is effected when the body segment of the fish crosses its longitudinal axis of motion. The greater efficiency of propulsion at this point also depends on the greater velocity of the body at the time when the longitudinal axis is being crossed. That the velocity of the gill varies in a similar manner I have observed, though only the employment of cinematographic methods would enable me to analyse it quantitatively. We thus have an interesting parallel in the forces involved in fish body movement and in insect gill movement. Both are operating on the " screw " principle (Gray, 1933, *a*, *b*, *c*) the fish performing a figure of eight, the gill an ellipse in each oscillation.

In the simplified system just described the gill would be expected to thrust water to the side equally in both up and downstrokes. That it does not do so will at once be understood when it is remembered that (i) the upper surface of the gill is convex and the lower, concave; (ii) the marginal filaments are flexed during the upstroke; (iii) the filament branches behave in the upstroke in such a way as to allow water to pass between them.

These facts introduce a feature of additional interest in that the principle of the oar in rowing is involved as well as that of the screw.

We may suppose that the description given here applies to any single gill of the left side and concerned with producing a flow from right to left, fig. 6A.

Its fellow of the right side would perform exactly the same movements. It would turn its undersurface to the right on the upward stroke and to the left on the downward beat. Also it would traverse an elliptical path in its vertical movement passing to the right of its median vertical axis of movement as it rises and to the left of this axis as it falls. Since one gill of a pair, however, is out of phase with its fellow, one gill would be in advance of the other in its action. Thus the effect of a gill, say, the right in a flow from right to left, is reinforced by the effect of the left gill, the latter being one-third of a complete oscillation behind the former.

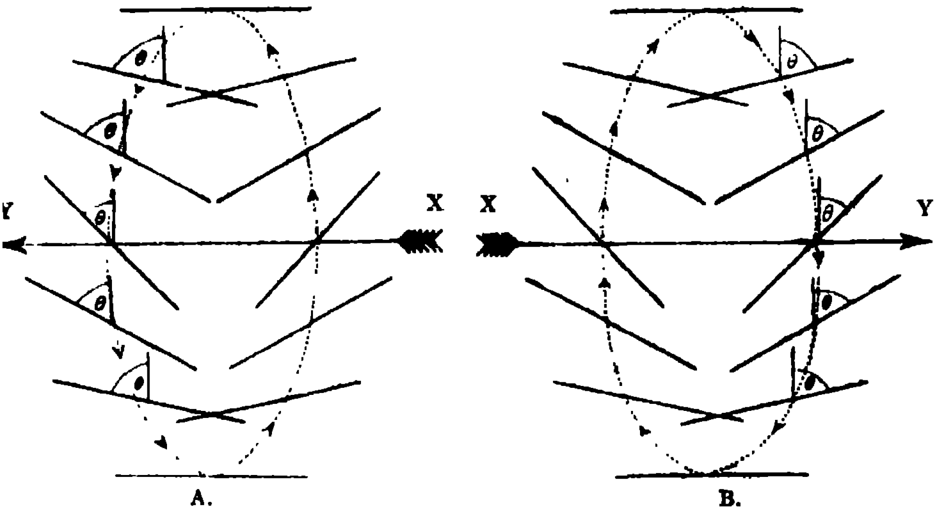

Fig. 6.—Diagram to show the elliptical path traversed by a gill in periodic up and down motion and the changing angle of the gill to its own path of motion. The arrows on the dotted ellipse indicate the actual path of motion; in A, of any gill concerned with a flow from right to left; in B, of any gill concerned with a flow from left to right. θ is the angle between the gill and a line at right angles to the main axis of flow indicated by the large arrow.

Considering further an animal with only the first pair of gills intact: if one of these gills is held still by means of a fine needle, the current for reasons given above still flows to the side. The same flow persists if the fixed gill be allowed to oscillate and the other one is held still. Now if this same pair of gills changes the direction of the current in the water, we find again that either gill is capable of producing a lateral flow in the new direction. Thus a single gill can produce a flow in either direction.

Proceeding then from our description of a single gill producing a flow from right to left; if this flow is reversed the path traversed is the reverse of that already described, fig. 6B; and the pivoting movements will take place in the opposite manner. Should both members of a pair be present we would again find one gill, viz., the right, reinforcing the effect produced by the left which precedes it in its oscillation.

Let us now consider the gills in one linear series. If from a complete individual producing, for example, a current from right to left, the gills of the right side be removed, the current usually continues to flow from right to left. The gills producing this flow move in metachronal rhythm from before backwards. They all exhibit the same type of pivoting movement, therefore the effect of any gill is reinforced by that of the gill behind it. But because of the rhythm, *forces of suction and compression are exerted on the water in the inter-gill spaces.* In fig. 7 by means of sine curves the oscillatory movements of the gills in this rhythm are shown on the left of the middle line. Reference to this figure indicates that a phase when gills 1 and 2 move apart (suction), is followed

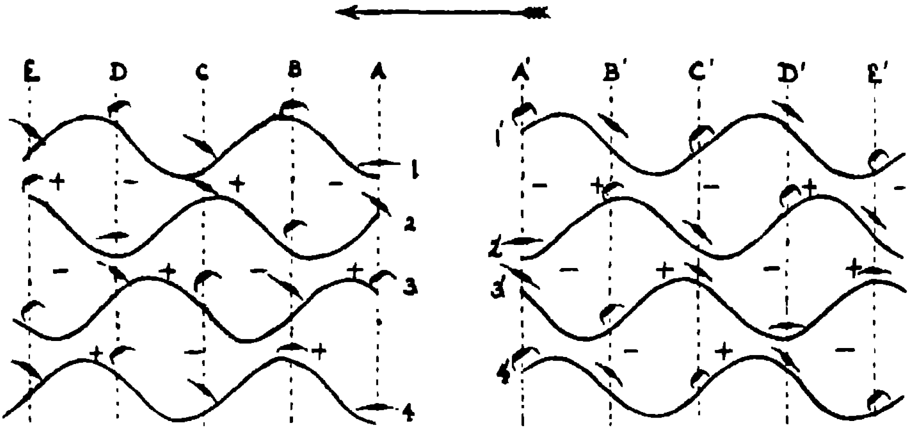

Fig. 7.—Sine curves 1, 2, 3, 4 (for the left) and 1′, 2′, 3′, 4′ (for the right) showing oscillatory movements of all the gills in producing a flow from right to left. The curves are drawn to read from the middle line, those for the left gills towards the left, those for the right towards the right side. The period of time involved in the movements indicated has been divided into four equal intervals ending at the dotted lines B, C, D, E, respectively for the left gills and at B^1, C^1, D^1, E^1, respectively for the right gills. Between adjacent gills periods of compression are marked by the sign +, and of suction by the sign −. The attitudes of the gills are given in the small figures above each curve. In falling the lower surface of each is turned to the *left* and the filaments are outstretched; in rising the lower side of each faces *right* and the filaments are flexed (see figures over curve I under B′ and A′ respectively). Positions of gills of one side correspond with those of the other under the same letter, A with A′, B with B′, C with C′, and so on.

by a phase when they approach each other (compression). When gills 1 and 2 are beginning a suction phase, Nos. 2 and 3 are in compression, and gills 3 and 4 are coming to the end of a suction phase. Periods of suction and compression alternate with each other down the series on the principle expounded by Cannon (1928), for the Crustacean *Chirocephalus*. That the forces invoked in these phenomena are significant in moving water is patent. We must conclude, however, from the capacity of a single gill to cause a side flow, that the effects that might be expected from rhythmical alternations of suction and compression between non-pivoting plates, are changed when the pivoting factor is introduced, the result being a side flow.

It has generally been found that an animal from which the gills of one side have been removed will continue for some time to deliver water to that side which retains its gills. This was so commonly noticed that it was at first wrongly thought to be the rule. A current in the reverse direction can, however, occur. If such an animal with a current flowing away to the side on which the gills are retained be fixed on its mutilated side, and a thick suspension of fine mud particles be introduced towards this latter side the current often reverses and flows against the proffered mud suspension. This reversal is accompanied by a reversal in the nature of the pivoting movement. No change of rhythm occurs.

Therefore, with one linear series of gills several principles are involved in current production (i) that which depends on the behaviour of the gill fringe in up and down movement—the bird's wing principle; (ii) the principle of the "screw"; (iii) the capacity of the gill to reverse this screw action by reversal of pivoting; (iv) that which concerns the alternation of suction and compression between the gills, caused by the metachronal rhythm.

The remarks made on the gills in one linear series apply equally to the gills in the linear series of the other side, see fig. 8 on the right.

There remains to consider the metachronal rhythms and gill movements when the gills of both sides are present. We have already seen, in considering the gill action in relation to the screw principle, that the effect of any gill is to reinforce the effect of its fellow—the result being a flow to one side. It may be that this is the only principle involved. There appears, however, to be the possibility of an additional factor.

Let us consider a single pair of gills, say the first, producing a flow from right to left. A point of some importance is the fact that the right gill, by means of its filaments, underlies the left in this case. Also, the phase difference between them in movement is such that the right has a leading phase relation to the left, of one-third of a complete oscillation. Since the right gill moves in advance of the left and underlies it by its filaments it is perhaps not unduly simplifying the case to think of the gills of a pair as being applied to each other so as to form a continuous membrane. This almost continuous unit may then be said to undergo a peristaltic wave from right to left. An initial suction phase on the right is followed by a suction phase on the left, a compression phase on the right having meanwhile come into existence. Thus on account of the nature of the rhythm across a pair of gills, suction and compression alternate across the two members of a pair so as to cause a current in line with the rhythm, *i.e.*, across the body segment. If the gills rose and fell in simple periodic motion in such a rhythm without pivoting, a peristalsis of this kind would cause a cross flow. We have already seen, however, that for a flow from right to left, both gills of a pair turn to the right in the upstroke and to the left on the downstroke, a movement which of itself can account for lateral flow. It would appear, therefore, that with a single pair of gills in action,

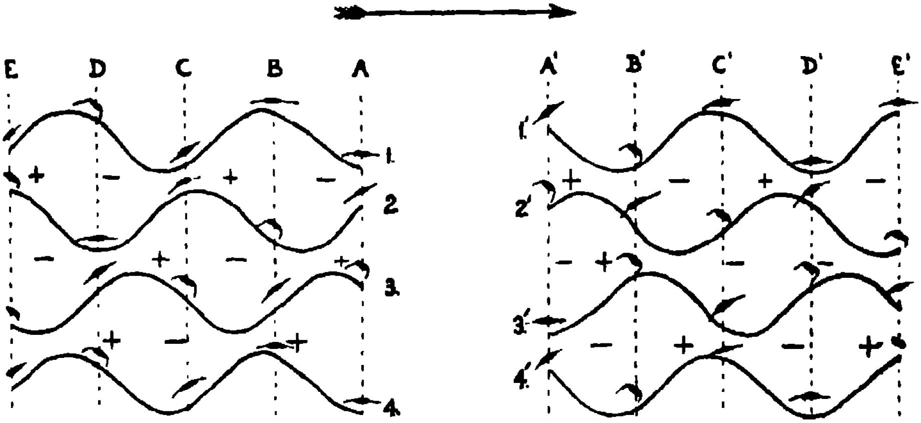

FIG. 8.—Sine curves 1, 2, 3, 4 (for the left) and 1′, 2′, 3′, 4′ (for the right) showing the oscillatory movements of all the gills in producing a flow from left to right. The curves are drawn to read from the middle line, those for the left gills towards the left, those for the right towards the right side. The period of time involved in the movements indicated has been divided into four equal intervals ending at the dotted lines B, C, D, E, respectively for the left gills, and at B′, C′, D′, E′, respectively for the right gills. Between adjacent gills periods of compression are marked by a + sign, and of suction by a — sign. The attitudes of the gills are given in the small figures above each curve. In falling the lower surface of each is turned to the *right* and the filaments are outstretched; in rising the lower side of each faces *left* and the filaments are flexed (see figures over curve I under A′ and B′ respectively). Positions of gills of one side correspond with those of the other under the same letter, A with A′, B with B′, and so on.

peristalsis and pivoting are complementary factors in the production of a flow of water across the body. The fact that the water enters a sub-gill space by passing between the gill filaments during the upstroke of the gill introduces a factor which does not seem seriously to affect the principles described above. When a single pair of gills with a current from right to left effects a reversal of flow, the gills come to rest momentarily. The originally underlying right gill now assumes an overlying position with regard to its fellow and at the same time the left gill assumes a leading phase relation to the right one. Further, the pivoting action which under the conditions of the first current was directed to the left side of the body is now reversed and directed to the right. Fig. 9 indicates diagrammatically the possible effect of peristalsis across a pair of gills where three successive phases of movement are shown.

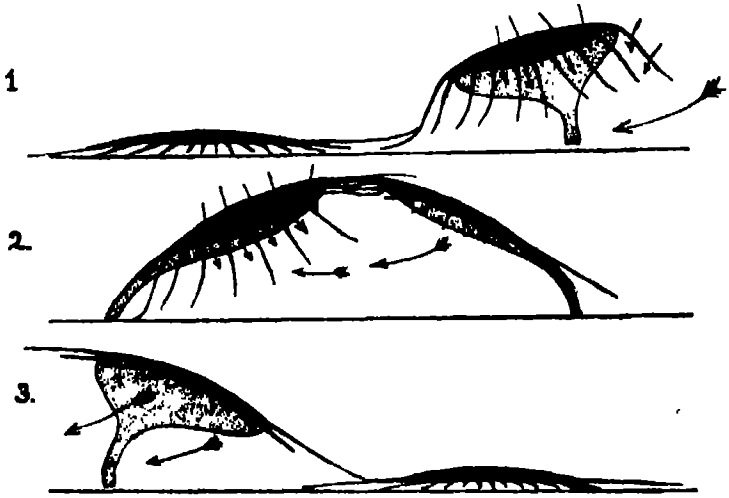

Fig. 9.—Diagram of a posterior view of a pair of gills in three successive phases of movement, 1, 2, and 3, producing a flow from right to left. The arrows indicate the direction of flow. In 1, the right and in 2, the left gill is rising. In 2, the right and in 3, the left gill is falling. It should be noted that the left gill overlaps the right, that the right gill is one-third of complete oscillation in advance of the left and that the pivoting movements of right and left gills are practically identical.

Let us now consider the first pair of gills in relation to those behind them. Since the axial rhythm is from before backwards the transverse rhythm across any segment must occur, in point of time, a little before that over the segment behind it. This being so, the flow over the body across a segment is reinforced by that over the one behind it. An examination of fig. 7, where oscillatory movements of the gills are shown by sine curves and the pivoting movements by the small sketches appended above each curve, will dispense with any lengthy explanation. In a flow from right to left the compression phase between gills 1 and 2 of the right side occurs during the suction phase of the left. In other words, the space between gills 1 and 2 of the left side is widening to receive water which can be forced into it by the compression phase between the right-hand gills. Add to this the fact that the gills are pivoting so as to assist this directional flow and the transverse flow where two adjacent pairs of gills are involved will be understood.

What applies to the first inter-gill space will apply equally well to the inter-gill spaces between gills 2 and 3 and gills 3 and 4.

The same principles will also hold for a current passing in the opposite direction; transverse rhythm, overlapping relations between members of pairs, and pivoting being of the opposite order to those described above, fig. 8. Actual reversal is always accompanied by changes in rhythm, overlapping and pivoting.

I have pleasure in thanking Dr. J. Gray, F.R.S., for the loan of a stroboscope during the early part of the work and for much helpful advice on its use. To Mr. K. G. Blair I am indebted for identifying the adult insects on emergence, and to Dr. C. G. Lamb for helpful discussion during the progress of the work.

Summary.

(1) The nymph of *Caenis*, by means of the oscillatory movements of four pairs of gills, produces a flow of water across the body from one side to the other. The current so produced is reversible.

(2) The gills rise and fall in periodic motion traversing an elliptical path, and make, by a pivoting action, an angle with their own path of motion. The metachronal rhythm in the movements of gills along each side of the body is from before backwards, but the gills of one side when in motion are always out of phase with those of the other. A transverse rhythm therefore exists across each pair of gills, this rhythm being in the direction of the water flow across the body. The transverse rhythm is reversed when the direction of the water current is reversed.

(3) The following factors are significant in determining transverse flow :—

(*a*) The filamentar fringe along the edge of each gill behaves differently in the upward and downward parts of each oscillation. Water passes between the filaments to the sub-gill space during each upstroke. In each downstroke

the filaments, by lying across each other, form a close mesh which by virtue of the smallness of its pores may be regarded as a membrane impervious to water. The gill is thereby rendered more efficient for moving water in the downward part of an oscillation than in the upward beat.

(*b*) Each gill turns its undersurface against the direction of flow in the upstroke and with the direction of flow in its downstroke. The principle of the screw involved, by virtue of the fact that the gill is inclined at an angle to its own direction of motion, is sufficient in itself to account for a flow across the body. A single gill can and does produce such a flow, and by its capacity to reverse the nature of its pivoting movements is able to cause a flow from right to left or from left to right.

(*c*) Since the gills act as plates moving in metachronal rhythm from before backwards, the alternating periods of suction and compression passing along any series of gills are significant in causing water movement.

(*d*) Any pair of gills by means of their filaments overlap, the one over the other in the middle line of the body. Upward movement of the underlying one begins first, and the transverse rhythm passing over them acts as a peristaltic wave driving water across. The effect of such a peristaltic wave over any segment is reinforced by that over the segment next behind it because of the metachronal rhythm which passes over the longitudinal series of gills from before backwards.

(4) Reversal of flow is associated with changes in (i) the method of pivoting of the gills ; (ii) their manner of overlapping as members of pairs ; and (iii) the direction of the transverse rhythm over the gills.

The axial rhythms never change individually, but the relation of the axial rhythm of one side changes with respect to the axial rhythm of the other thus causing a reversal of the transverse rhythms involving gills forming segmental pairs.

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The Measurement and Analysis of Colour Adaptation Phenomena.

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Introduction.

The phenomenon with which this paper deals is one that can be readily observed without any special apparatus at almost any time and is, indeed, familiar to all those concerned with visual matters, whether scientists, artists or others. Thus when the eye gazes at a patch of light for a short time the sensitivity of the eye is modified and the appearance of a coloured object will be changed from its normal appearance to an extent dependent on the nature of the initial patch of light. The object of the experiments described below has been to measure such changes under various conditions: for different intensities and colour of the initial patch; for varying times of viewing this patch; and for a test object whose colour and intensity is also variable; and finally to attempt to relate the results to the adaptation process and the three-colour response curves of the eye.

Fig. 1 is a schematic diagram illustrating the action of the visual mechanism. To the left of H, a, b, c, d, ... represent the visible radiations of various wave-lengths and amounts of energy, while to the right of H the diagram represents the visual chain leading to the brain. At some point P along this chain the visual response is divided into three independent paths A, B and C. Each of the radiations a, b, c, d, ... may have been modified into stimuli a', b', c', d', ... but each radiation will still have its corresponding stimulus at P and each will, in general, contribute something to the three responses A, B, C.

This analysis admits, for the sake of generality, the possibility that a, b, c, d, ... might each react to produce its own independent nerve response, the magnitudes of these responses being represented by a', b', c', d', ...; these nerve impulses themselves can then be regarded as stimuli that react at P to produce responses along A, B, and C. In practice it is very difficult to conceive that a', b', c', d', ... can be different in nature from a, b, c, d, ..., although their relative magnitudes may have been altered by absorption in some intermediate pigment.

Now it is well known that when the eye is directed to a fairly bright area of light, the sensation is much greater during the first two or three seconds than it is after two or three minutes. This is generally ascribed to a reversible photochemical action in which some substance is decomposed by the light at a decreasing rate until the rates of decomposition and regeneration just balance. It is of considerable theoretical interest to know whether this process takes place at H, at P, or between H and P, say at M; or it may possibly occur as three independent processes along A, B and C, say at N. A fundamental point in this connection is the experimental fact that when a colour match (viewed at the fovea) is made between a test colour and a mixture of three physical primaries, say a red, a green and a blue, then if the eye is subsequently allowed to view a bright patch of light of any colour and become adapted to that higher intensity level, the colour match will still appear to hold when the eye is re-directed to the two patches. This is true at least over a wide range of intensity. Hence if the adaptation process occurs at M, it can only act by reducing the stimuli, of all wave-lengths, by the same factor. This can be compared to the introduction of a rotating sector in the beam just before it enters the eye. If any action took place which produced a change in the relative values of a', b', c', d', ... it would be equivalent to the introduction of a coloured glass into the beam, instead of a sector. It is easily demonstrated that this would, in general, affect the appearance of beams having the same

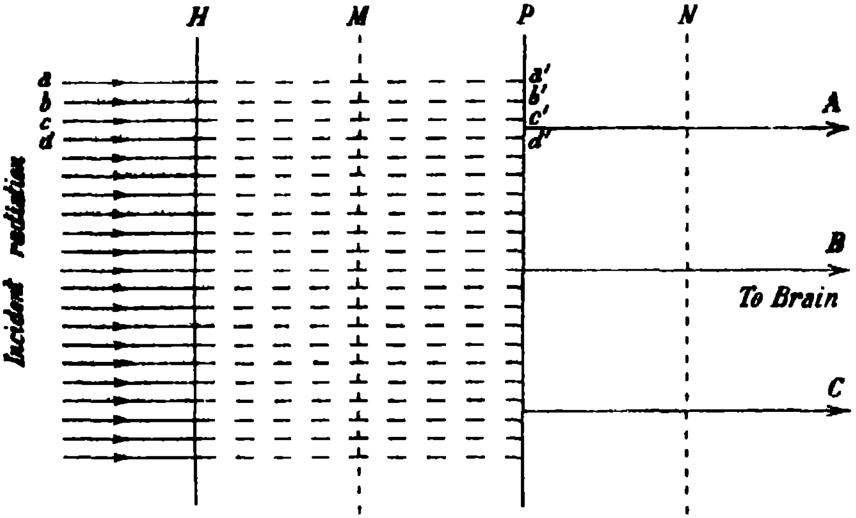

FIG. 1.—Schematic diagram to illustrate action of visual mechanism.

colour but different spectral composition in different ways and a match between two such beams would accordingly be destroyed.

We therefore arrive at the very important conclusion that any adaptation process produces either uniform reductions of all the stimuli to the left of P or specific reductions of the three responses between P and the brain, or both. And we can anticipate that measurements of the effects of colour adaptation should provide evidence to determine which of these alternatives is the correct one ; to indicate the nature and laws of the process ; and possibly to determine the proportions in which *a*, *b*, *c*, *d*, ... each stimulate the three response paths, that is, to determine the three spectral response or excitation curves.

Apparatus and Technique.

The apparatus used was the trichromatic colorimeter designed by the author, in which three spectral radiations are used as primaries and mixed in proportions that can be controlled by the observer to match any given test colour. The optical system is shown in fig. 2. This is similar to the system described elsewhere (Wright, 1927–28) except that while the test colour is still viewed by the right eye, the other patch, the mixture of the three primaries, is now viewed by the left eye.

The function of the apparatus is briefly as follows : Light from a pointolite lamp S is focussed on the slit of a collimator and from the collimating lens C a parallel beam passes through two 60° dispersing prisms and the top half of the beam is brought to a focus in a spectrum W_1 by the lens T_1. The lower half of the beam is reflected by the prism R_1 and is focussed in a second spectrum at W_2. In W_1 a small roof prism, T.C., reflects a narrow portion of the spectrum back through the optical system along a lower level until it is reflected by the prism D_1. In spectrum W_2 three small prisms R, G, B reflect back three portions of the spectrum through the dispersing prisms, the dispersion is neutralized, and a parallel beam composed of a mixture of the three radiations is reflected by D_2. The beam from D_1 passes through an aperture K_1 and is focussed by O_1 at the exit-pupil E_1, while that from D_2 passes through a periscope prism P, then through K_2 and is brought to a focus at E_2. The right eye is placed at E_1 and the left at E_2. K_1 and K_2 each subtend approximately 1° × 2° at the eye and their separation and that between E_1 and E_2 are adjustable to suit the observer. In this way it can be arranged so that, by suitable convergence of the two eyes, the two patches are brought into apparently contiguous positions, while at the same time each patch is focussed on the fovea of the eye concerned. Small circular apertures (diameter = 0·040 inch) are used as exit-pupils at E_1 and E_2 and variations in measurement due to changes in the iris of the eye are thus eliminated. The monochromatic patch seen at E_1 serves as the test colour; its mean wave-length can be varied by moving the T.C. prism through the spectrum W_1. The patch at E_2 was, throughout the present work, illuminated by a mixture of three radiations of wave-lengths 0·65 μ, 0·53 μ and 0·46 μ. These radiations acted as the instrument primaries and their relative intensities could be controlled by the observer by manipulation of photometric wedges, H, mounted in front of R, G, B.

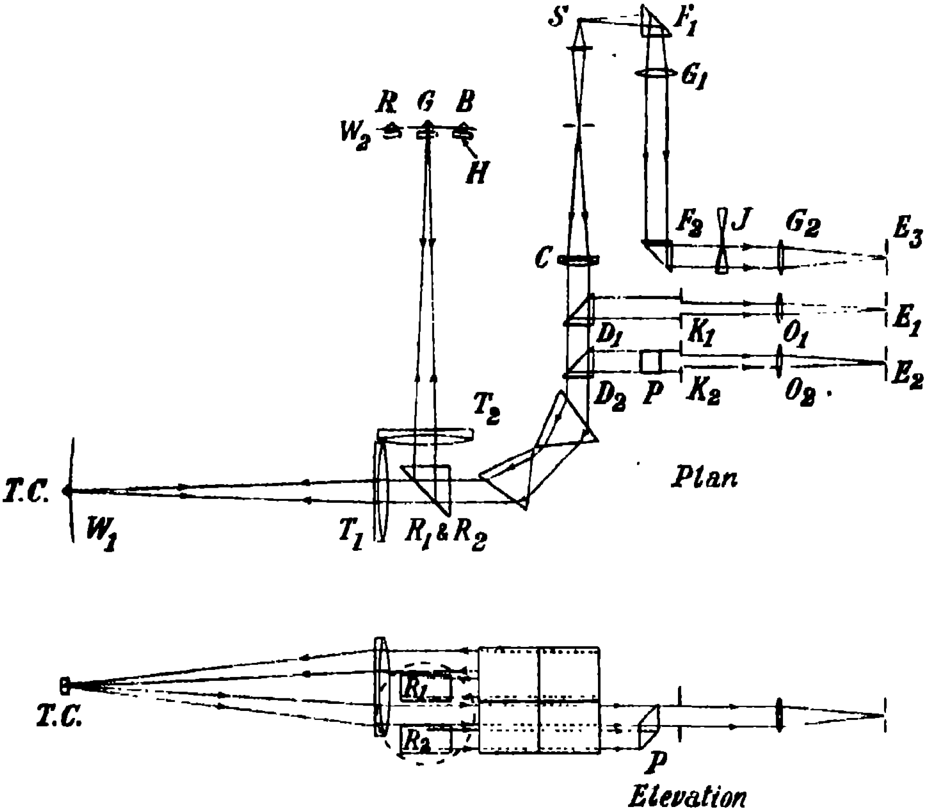

FIG. 2.—Optical system of trichromatic colorimeter.

For some of the experiments, the patch to produce the adaptation was obtained direct from the pointolite lamp via the prism F_1, the lens G_1, the prism F_2 and the lens G_2. An image of the tungsten ball of the lamp was formed in a small aperture (diameter = 0·035 inch) at E_3. When the eye was placed at E_3, the lens G_2 appeared as a uniform patch of light of 6° angular subtense. Neutral filters and coloured Wratten filters could be used to control the intensity and colour of the patch and in addition a series of fixed aperture sectors could be rotated at J to vary the intensity by known amounts. The energy distribution of the light approximated to that of a black body radiation of 2100° K.

A monochromatic patch for the adaptation was also provided by mounting a narrow mirror in the spectrum W_1 and reflecting the light above the main apparatus into a lens, not shown in the diagram, fixed some distance vertically above O_1. When the eye was placed at the focus of this lens a circular patch of monochromatic light, of about 8° angular subtense, was seen.

In addition to the monochromatic test colour from the T.C. prism, a white test colour could also be used. This was obtained by illuminating a white magnesium oxide screen by light from a 1000 watt tungsten filament lamp. The colour temperature of the lamp was approximately 2500° K., but the position of the white point in the colour triangle was measured in each experiment. In all the work the intensities of both the pointolite lamp and the filament lamp were kept constant throughout a series of experiments by accurate control of the voltage supply.

The experiment is essentially quite simple. After a period of dark adaptation lasting about 30 minutes, the test colour, as seen by the right eye, is matched against a mixture of the three instrument primaries as seen by the left eye. The wedge readings are then noted by a second experimenter who is operating the apparatus. The observer now puts his right eye at E_2 and gazes steadily at the 6° patch of light for, usually, 3 minutes. In the meantime the left eye is unilluminated. At the end of the 3 minutes the head is moved rapidly back to the colorimeter so that once again the test colour is viewed by the right eye and the three primaries by the left. The previous match no longer holds and the change is measured by adjusting the intensities of the primaries as quickly as possible until a correct match is obtained. The observer announces when this has been done so that the operator can again record the readings and in addition, with the aid of a stop-watch, the time at which the observation was made. The observer's right eye is in the meantime recovering from its depressed sensitivity and a fresh match must be made to determine the course of recovery. In practice it is found possible to record observations about once every 30 seconds, the first match usually being made about 10 or 15 seconds from the end of the adaptation. Each experiment is repeated to enable a mean curve to be plotted. Sometimes the repeat experiment was not conducted immediately after the first adaptation, but a series of experiments were made and then the whole series repeated.

The validity of the experiment rests on the assumption that the sensitivity of the left eye remains unchanged throughout and can therefore be used as a standard of reference. This is not strictly true, but it can be readily observed that the change produced in the right eye is many times greater than that produced in the left. It would undoubtedly be best to allow for the effect, were that possible, but the error introduced by not doing so is of no significant magnitude.

The presence of superposed after-images of any appreciable intensity would also tend to confuse the interpretation of the results, but with the possible exception of the blue, no effect of this sort has been observed. The reason for this is the relatively low intensity of the after-images compared to that of the test colour. That no disturbing effect is present is shown, for example, by the fact that reversals from positive to negative after-images cause no alteration in the appearance of the test colour.

The units in which the observations are recorded are based on matches of a monochromatic yellow, of wave-length 0·5825 μ, in which the amounts of red (0·65 μ) and green (0·53 μ) are taken as equal, and on a match of 0·4940 μ. in which the amounts of blue (0·46 μ) and green (0·53 μ) are considered equal. This method of obtaining the units and its advantages for visual research, have already been described by the author (Wright, 1929). The relative brightness of unit quantities of the three primaries, for the author's eye, are as follows:—

L_R (0·65 μ) : 0·648

L_G (0·53 μ) : 1·000

L_B (0·46 μ) : 0·051.

The retinal intensity in photons of both the test colour and the adapting radiation are given in the results recorded below. These were obtained from measurements on a Hefner lamp, but must be regarded as of an approximate nature only. The experiments have usually been arranged so that the particular point under investigation was dependent on relative rather than absolute measurements of intensity. It is nevertheless important to know the level of intensity at which particular experiments have been made, both for comparison with previous work and as a guide to future investigations.

All the observations recorded have been made by the author. The time to acquire facility in making binocular matches and the necessity of continual checking of the results by repeating the experiments have made it impossible to extend the research to include detailed observations by other observers.

Mr. F. H. G. Pitt, however, has repeated a number of representative experiments and his results have agreed in their general characteristics with those of the author. Owing to the limitations of space, only the essential results obtained by the author have been given; in all, some 5000 matches were recorded.

Typical Recovery Curves.

In fig. 3 the results for a typical experiment are shown. The initial match on the test colour, which happened to be a white (retinal intensity about 200 photons), gave the amounts of the three primaries as

$$R = 1750, \quad G = 1900, \quad B = 970.$$

While the relative magnitudes of these quantities have the significance defined above, the absolute magnitudes are quite arbitrary, depending on the calibration curves in use at the time of the experiment, the presence of auxiliary filters in the apparatus and so on. It should be noted that R, G, B will always be used to indicate the values of the initial match made on the test colour.

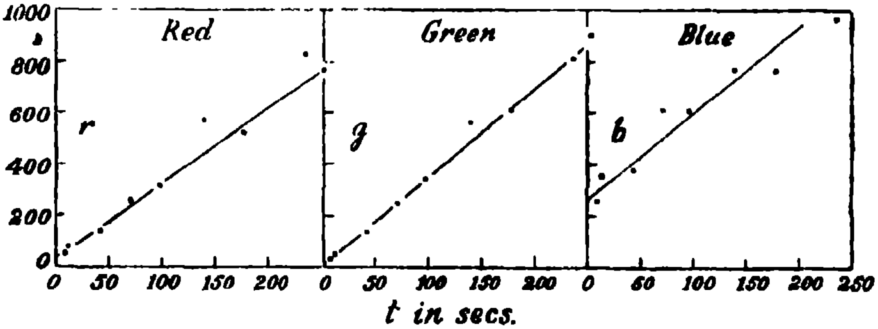

Fig. 3.—Recovery curves using an adaptation radiation of wave-length 0·56 μ, and intensity 1500 photons with a white test colour, R = 1750, G = 1900, B = 900, of intensity 200 photons.

As adapting radiation a monochromatic patch of mean wave-length 0·56 μ was used with an approximate retinal intensity of 1500 photons. Three minutes adaptation was allowed. Measurements of the recovery of sensitivity were made in the manner already described and the whole experiment repeated. In fig. 3 the ordinates give the time, *t*, in seconds from the end of the adaptation, while the abscissæ represent the intensities *r*, *g*, *b* required by the dark adapted left eye to match the test colour as seen by the light adapted right eye. In the remainder of the work, *r*, *g*, *b* will always be used to represent measurements made on the test colour during the recovery from the adaptation; where a suffix is added, this indicates, in seconds, the time from the end of the adaptation, at which the measurements were made.

The amount of scattering of the points gives an idea of the reproducibility of the physiological condition of the eye and of the difficulty of the observations. With practice the matches can be made fairly easily, although the experiment is rather liable to disturbance from extraneous influences. The scattering is especially noticeable in the blue, on account of its low luminosity and for this reason unless the blue values were particularly required owing to the nature of the experiment, only the red and green values were recorded. This had the advantage that the operator had to record only two, instead of three readings each time a match was made, thus saving time and allowing more matches to be made; in addition the observations were centred on the most reliable quantities.

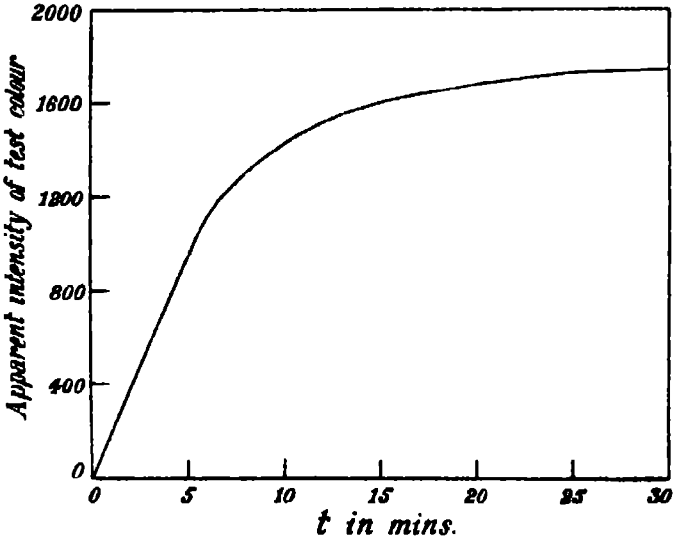

Fig. 4.—Complete recovery curve after light adaptation.

The first point to note in connection with fig. 3 is that although the blue has returned to its original value after the recovery has lasted about 200 seconds, the red and green are still considerably below that level. This is a characteristic that is found in almost every experiment, namely, the rapid and complete recovery of the blue and the more or less rapid but incomplete recovery of the red and green. The recovery of the latter is, of course, completed, but at a much slower rate. The complete course of recovery is as shown in fig. 4;

for intensities of adaptation between 1000 and 10,000 photons, 30 minutes may elapse before the original value is regained Yet visually the depression of the red and green at $t = 200$ or 250 seconds is relatively slight and insignificant compared to the much greater depression immediately after the adaptation. We can anticipate the discussion given later to state that the rapid recovery represents the recovery from the light adapted state to a mean level of adaptation, while the prolonged recovery measures the development of the enhanced sensitivity normally associated with dark adaptation. Beyond that we need only at present note the delay in the complete recovery and at the same time point out that the main process is in fact completed after 200 or 300 seconds. Our attention will be largely devoted to this portion of the curve.

The most striking feature of fig. 3 is the linear nature of the curves. This has been repeatedly confirmed by numerous experiments, except when very high intensities of adaptation are used. It may, perhaps, be objected that for small values of t, say, 5 or 10 seconds, a large percentage error in r or g would produce only a negligible displacement from a straight line, but a comparison with the non-linear curves obtained with high intensities shows the striking difference between the two and indicates the probability that if the recovery curves were not linear below 10 seconds, the non-linearity would still be apparent after that time. Moreover, in a number of curves given later the intensity at 10 seconds is considerably higher than in fig. 3, and a much smaller displacement would be noticeable. It is, of course, very easy to imagine that in the first second or two, the recovery processes might start, perhaps, with a sudden burst of activity or, more probably, a short delay might occur before the action commenced. In neither process, unfortunately, would it be possible to measure such effects in these experiments, but the conclusions that have been arrived at are not greatly affected by possible secondary effects of this sort.

Within the limitations implied above, the lines can be produced back to intersect the axis at $t = 0$ and the values of r, g and b at this point will measure the sensitivity of the eye at the time when the adaptation is just ended. These values will be of particular significance in measuring the depression of sensitivity for varying time and varying intensity of adaptation.

It may be noticed that the green curve intersects the axis very close to a value of $g = 0$. This is of no special theoretical consequence; in fact, for some combinations of test colour and adaptation, the curve may cross the axis to give negative values of g. It is then simply an indication that the colour seen by the right eye lies outside the colour triangle made by the three instrument primaries used in the left eye. It is of some practical importance as it tends to limit the number of combinations of test colour and adapting radiations that can be used, since a negative amount of primary is not physically available. Incidentally, even if there were no other reason, the presence of negative quantities illustrates the desirability to record the recovery in terms of r, g and b, rather than by $\log r$, $\log g$ and $\log b$. It will be shown later that r, g and b are the significant values in which we are interested.

Variation of Time of Adaptation.

Considerable theoretical interest attaches to the determination of the effect of varying the time of adaptation and, in addition, it is very important for the rest of the work to know how long the eye must be light adapted before the photochemical balance between decomposition and regeneration is reached.

In fig. 5 (*a*) the recovery curves for adaptation times ranging from 10 seconds to 3 minutes are shown. In this experiment a monochromatic test colour, wave-length 0·5825 μ and retinal intensity 240 photons, was matched in the left eye by

$$R = 1854, \quad G = 1854.$$

A white adaptation, intensity 2000 photons, was used.

As might be anticipated, the depression of sensitivity was greater the longer the time of light adaptation. The depression values at $t = 0$ are plotted in fig. 5 (*b*) against the time of adaptation to give a curve which is a record of the course of the light adaptation process. It is interesting to note how very rapidly the sensitivity is depressed and to realize that an action of this type occurs whenever the eye is directed to an area appreciably brighter than the one to which it had been previously adapted. The effect is not particularly noticeable under normal conditions when the eye may pass rapidly from one object in a room to another; then the adaptation level is determined by the average brightness of the objects in the room, and the eye will not usually rest on any one object long enough to be adapted to its particular level. But when entering a brightly lighted room from a dimly lighted one, the visual response is much greater in the first few seconds than after 2 or 3 minutes. Fig. 5 (*b*) in effect illustrates the course of this process. It is, however, not to be expected that this curve would be of much value in determining the actual photochemical process taking place. A number of equations could be developed each of which would fit the observations within the accuracy warranted by the

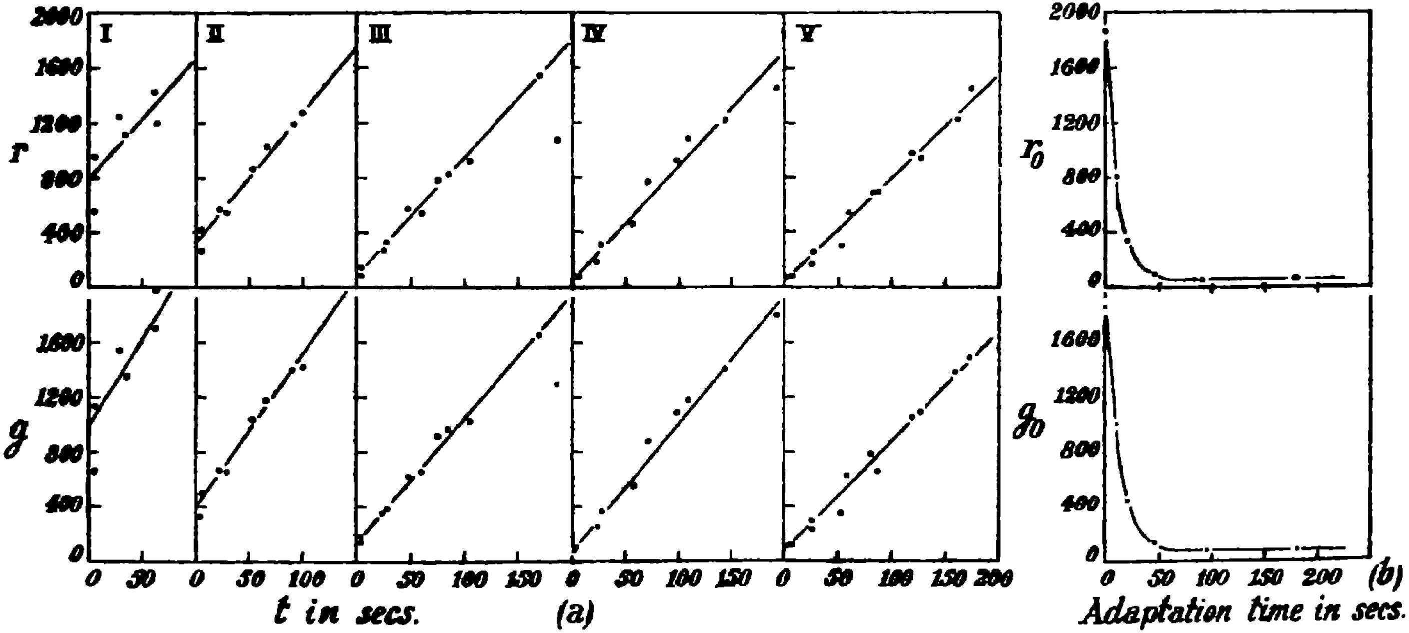

FIG. 5.—Effect of variation in time of adaptation, using white adaptation, intensity 2000 photons, and yellow test colour, wave-length 0·5825 μ, intensity 240 photons. Time of adaptation: I, 10 secs.; II, 20 secs.; III, 45 secs.; IV, 90 secs.; V, 180 secs.

experiment. In this experiment, the observations for the shortest fatigue are very widely scattered. Variations of this sort are not uncommon, as a slight hitch in the procedure or a momentary uncertainty may upset the observations to an unpleasantly large degree.

An important feature of fig. 5 that should be noted here, although it will not be discussed until later, is the fact that the recovery curves are nearly parallel to one another. This is somewhat unexpected, but it has been repeatedly confirmed and a similar result is found when the intensity of the adaptation is varied. This at least satisfies one condition that we should expect to hold, namely, that at the same level of sensitivity the rate of recovery is always the same, whatever the previous depression has been. This would not be so if $\log r$, etc., were plotted against t.

The practical point in which we are interested, the time to allow to ensure that the balance point in the adaptation process has been reached, is answered by these results. Evidently a period of 3 minutes light adaptation should be adequate for most purposes. It is not to be assumed that after 3 minutes the visual mechanism is in a perfectly stable condition. This is very unlikely, but any changes that do occur are certainly both small and gradual. In the rest of the work 3 minutes adaptation has been used.

Variation of Test Colour and Determination of the Fundamental Response Curves.

(*a*) *Variation of Test Colour.*—In the experiments already described the test colour used was selected because it had a convenient proportion of red to green and was of a suitable intensity. It must now be shown what effect a variation in either intensity or wave-length of the test colour has on the recovery curves.

In fig. 6 the recovery curves for the red and green primaries are given for a white adapting light (intensity 700 photons) and a yellow test colour. Three intensities of test colour were used, the highest being 10 times brighter than the lowest. The reduction in sensitivity can be compared for the three by extrapolation to $t = 0$. These values, r_0 and g_0, are compared to the initial matches, R and G, in Table I. It will be seen that g_0/G has an approximately constant value, while the values for r_0/R are all of the same order.

There are probably two main reasons to account for the deviation from a constant value. In the first place for the highest intensity of test colour, the difference of intensity between the test colour and the adapting radiation is not very great, so that immediately the matches are attempted the left eye will tend to become light adapted to an appreciable extent and a greater value of r or g will be required. Hence one condition for successful experiments is

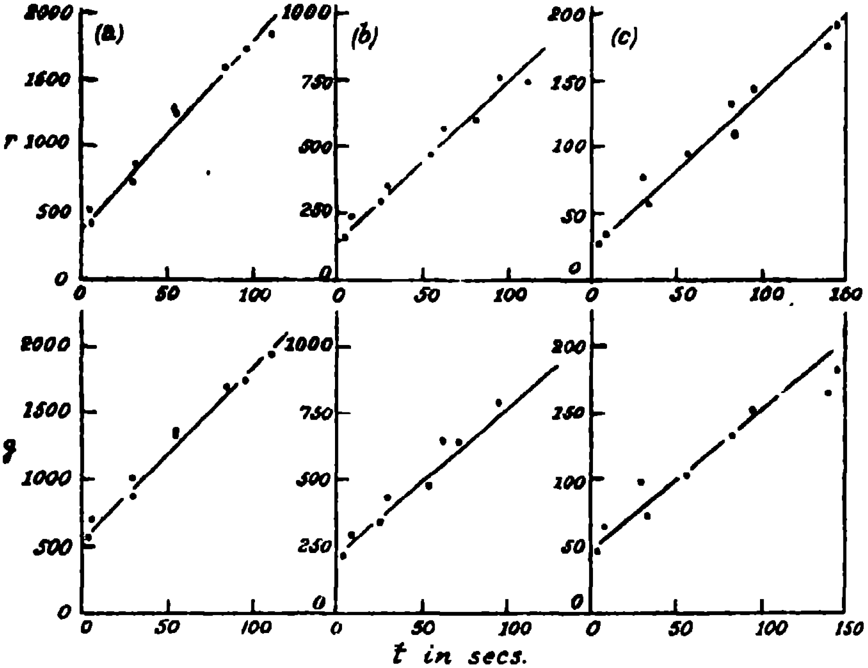

Fig. 6.—Recovery curves using white adaptation, intensity 700 photons, and the three intensities of test colour, wave-length 0·5825 μ. (*a*) Intensity of test colour, 360 photons, R = G = 2510. (*b*) Intensity of test colour, 160 photons, R = G = 1120. (*c*) Intensity of test colour, 36 photons, R = G = 250.

Table I.

Intensity of T.C.		r_e.	g_e.	r_e/R.	g_e/G.
R.	G.				
360 photons					
2510	2510	380	600	0·151	0·239
160 photons					
1120	1120	150	230	0·134	0·214
36 photons					
250	250	25	55	0·100	0·220

that the test colour should be of low intensity in comparison with the adapting radiation; the effect of the light adaptation in the left eye will then be negligible in comparison with the right. The other source of variation is the inherent inaccuracy in extrapolation to $t = 0$. This should not be very great in fig. 6, but with higher intensities of adaptation and correspondingly smaller values of r_0 and g_0, the percentage error may be very appreciable. For this reason it is more reliable to compare readings at some time shortly after the recovery has started, say at $t = 20$ seconds.

This has been done for the same test colours used above but with an intensity of adaptation (3500 photons) five times as great. The values obtained at $t = 20$ seconds are given in Table II. In this case the agreement for the three test colours is more convincing and is, in fact, as close as could be expected in view of the difficulty of the observations.

Table II.

Intensity of T.C.		r_{20}.	g_{20}.	r_{20}/R.	g_{20}/G.
R.	G.				
360 photons					
2510	2510	320	420	0·127	0·167
160 photons					
1120	1120	150	175	0·134	0·156
36 photons					
250	250	35	47	0·140	0·188

The implication of this result is that if an intensity i caused the same sensation in the dark adapted eye as an intensity I in the light adapted eye, then a reduced value of i, say $i/10$, would produce a reduced sensation in the dark adapted eye that would be matched by an intensity 1/10 in the light adapted eye. In addition to other experiments similar to the above, this was also checked directly by measuring the recovery from a given adaptation in the ordinary way and then repeating the experiment with a sector introduced so that it cut down the beams equally in the right and left eyes. The curves obtained should coincide; a very close agreement was found, as shown in fig. 7.

It is evident, then, at least for intensities that are small in comparison with the degree of adaptation, that if we have a match between intensities of i and I measured at different sensitivity levels, the match will also hold between αi and αI. This relation has been postulated before as a "law of coefficients" (von Kries, 1904), although the experimental evidence on which it was based does not seem very adequate. So far as can be seen, the only reason that the relation could not be said to hold for all intensities, large or small, is that a large intensity would immediately produce a change in the state of adaptation. If observations could be made before this change occurred the same relation would presumably be found to hold.

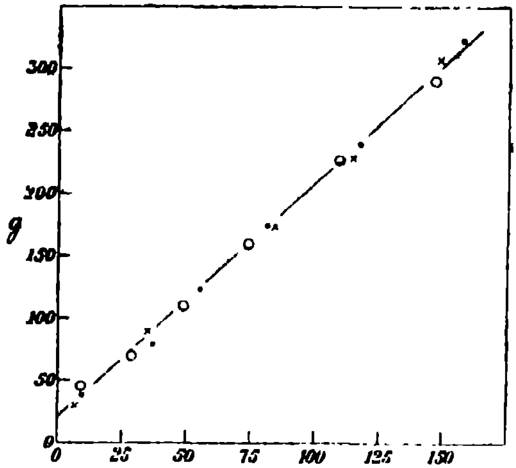

FIG. 7.—Green recovery curve using white adaptation, intensity 1200 photons, and yellow test colour, wave-length 0·5825 μ. G = 544. Results are shown for three levels of intensity of test colour and comparison beam, obtained with rotating sectors to intercept beams in both the right and left eye. Intensity of test colour ● 360 photons; ○ 60 photons; × 36 photons.

An important corollary to observe is that these observations provide strong circumstantial evidence that r, g and b are direct measurements of the response, as opposed to other functions such as $\log r$, $\log g$, $\log b$. When the sensitivity of a mechanism is reduced, the response aroused by any given stimulus is usually reduced in a ratio independent of the magnitude of the stimulus. This will only hold in the present case if we measure the response in terms of r, g and b.

Now suppose R′, G′ and B′ are the hypothetical stimuli that produce responses along A, B and C, fig. 1, respectively. Then a reduction in sensitivity produced by light adaptation will, for a test colour that stimulates A alone, produce an intensity depression of R′ but no colour change; similarly if B or C are stimulated alone. Then if, in a given state of adaptation, R′ were reduced to r' and G' to g', as each response is independent, $(R' + G')$ would be reduced to $(r' + g')$, $(2R' + G')$ would be reduced to $(2r' + g')$ and so on. In other words the geometry of the colour triangle holds equally well for the depressed eye as for the eye in its normal state. This also follows independently from the fact that a three colour match made in one eye remains a match even when the eye is light adapted over a wide range of intensity. Hence if the effect of a particular colour adaptation on the colours of the three instrument primaries R, G, B is known, then the effect on any colour in the colour triangle can be calculated.

This deduction is also given by von Kries (1904), but it seemed desirable to check it experimentally and the results of one test are given below. Three monochromatic test colours, 0·65 μ, 0·5825 μ and 0·53 μ, were used. For the adaptation the beam direct from the pointolite was used with a green Wratten filter interposed (intensity 1200 photons, spectral distribution not determined). The values of r, g and b for each test colour were read off from the recovery curves at $t = 20$ seconds to give the results shown in Table III.

Table III.

Wave-length and intensity of T.C.	Observed values.				Reduced values.			
	R.	G.	r_{20}.	g_{20}.	R.	G.	r_{20}.	g_{20}.
0·65 μ, 60 photons	1746	—	270	—	1000	—	155	—
0·5825 μ, 170 photons	1941	1941	350	212	1000	1000	180	109
0·53 μ, 70 photons	—	1337	55	140	—	1000	41	105

The left half of Table III gives the results for the actual amounts of the test colour that were used; on the right-hand side the data have been reduced to amounts of R, G and B that can be directly compared. In these units the values of r_{20} and g_{20} for the 0·65 μ and 0·53 μ test colours should, when added together, give the r_{20} and g_{20} values for 0·5825 μ. The agreement is remarkably close. Similar experiments have confirmed that result, although the margin of experimental error has usually been greater than here.

The effect of varying the test colour is thus reduced to a matter of colorimetric additions and subtractions. If the intensity of the test colour is reduced, the recovery curves are reduced by the same amounts throughout;

if the colour is changed, the change can be calculated provided the effect of the adaptation on three arbitrarily selected primaries is known. These conclusions must not be regarded as absolutely rigid; they appear to hold at least very approximately over a mean range of intensity, but at very low intensities after-images will be of comparable and disturbing magnitudes.

(*b*) *Determination of the Fundamental Response Curves.*—It has been mentioned above that if we could find a test colour that stimulated only one of the responses then no matter how the sensitivity of the eye was depressed, no colour change could be evoked; for a colour change necessitates a change in the proportions of at least two of the responses and since only one is being stimulated, no qualitative change can be produced.

Further, if the positions in the colour triangle of these three fundamental stimuli were known, then the fundamental response or excitation curves could be computed by combination with the trichromatic mixture curves and the luminosity curve. Hence to determine these response curves it is only necessary to find those theoretical stimuli that, no matter how the sensitivity of the eye has been depressed, evoke sensations that are modified in intensity but not in colour.

A direct investigation of the spectral radiations for this property can be made very easily. The patches in both the left and right eye can be illuminated with the same spectral radiation and the right eye can then be adapted with any coloured light. For a comprehensive test red, green and blue adaptations should all be used. At the end of the adaptation the two monochromatic patches are again viewed and if no colour change has been induced in the right eye, it should be possible to obtain a match merely by reducing the intensity of the light in the left eye. It should be noted that the term "colour change" is intended to include a change in saturation as well as a change in hue.

If such a series of experiments is conducted, it is found that in two regions of the spectrum there are radiations in which only very small changes are induced. The first of these is in the extreme red, from 0·70 μ to the end of the spectrum. With this test colour, with any wave-length of adapting light, only an insignificant colour change is induced. Sometimes a small amount of blue, the origin of which is discussed later, is superimposed, but while it is of interest it is of negligible proportions. For all practical purposes it can be concluded that the extreme red stimulates only one of the responses. The point in the colour triangle corresponding to this region can therefore be taken as R′, the red fundamental stimulus; its equation in terms of R and G has been taken from the author's colour mixture curves obtained in previous work and is given by

$$R' = 1\cdot036R - 0\cdot036G.$$

The other region of the spectrum in which practically no colour change is induced is in the neighbourhood of 0·46 μ. For either a red, green or blue adaptation the change is very slight; the major effect is an increase or decrease in saturation, but little error would be introduced if 0·46 μ itself were taken as the second of the fundamental stimuli. Nevertheless, it is shown below that a more accurate determination of the point presents no great difficulty and its precise location has been found.

In the green region of the spectrum the colour change is very much more marked. Thus after adaptation with red, a green at 0·53 μ will become a purer, more saturated green, while after green adaptation it will appear yellow-green or yellow. It has been shown above that once the effect on three primaries is known for a particular adaptation, the change produced on any given point in the colour triangle can be computed. It has now to be shown that the inverse process, the location of a point that has a given colour change, for our purposes zero change, is also possible.

This can be shown most readily by using the data given in Table III. From this table we find that

1000R after adaptation become 155R,

and

1000G after adaptation become (41R + 105G).

If we assume that the trichromatic coefficients of the fundamental stimulus are given by $(xR + yG)$, then after adaptation $(xR + yG)$ becomes

$$\frac{x}{1000}(155R) + \frac{y}{1000}(41R + 105G),$$

$$= (0\cdot155x + 0\cdot041y)R + 0\cdot105yG.$$

As this is to be the point giving no colour change, the ratio of the R and G coefficients before and after the adaptation must be the same, hence

$$\frac{x}{y} = \frac{0\cdot155x + 0\cdot041y}{0\cdot105y},$$

giving $x = -0\cdot82y$.

The green stimulus is therefore represented in the colour triangle by the point y $(-0\cdot82R + 1\cdot0G)$. The location of the point has simply involved the use of the normal properties of the colour triangle. The only approximation that has been made is the omission of the blue. It has been found that for test colours between 0·65 μ and 0·53 μ the changes are almost entirely a function of two variables, the red and the green. A small quantity of positive blue is evoked by some adapting radiations, but the amount is negligible in comparison with the changes induced in the other two primaries. It may be due to a superimposed after-image or be associated with other abnormalities of the blue found later.

The sample calculation has been particularly easy, owing to the choice of an adapting radiation that caused no colour change in R. In some experiments a spectral green was used for the adaptation and this caused R to become too saturated to measure. It is then necessary to use, say, 0·53 μ and 0·5825 μ as test colours and obtain the change in R by subtraction. Similarly, if a red adaptation is used, the green test colour will move outside the colour triangle and be impossible to match, but the changes in 0·65 μ and 0·5825 μ can be measured and the former subtracted from the latter to give the change in 0·53 μ.

Table IV.—Green Stimulus Coefficients.

	$y(-0{\cdot}70R + 1{\cdot}0G)$
	$y(-0{\cdot}80R + 1{\cdot}0G)$
	$y(-0{\cdot}64R + 1{\cdot}0G)$
	$y(-0{\cdot}55R + 1{\cdot}0G)$
	$y(-0{\cdot}82R + 1{\cdot}0G)$
Mean	$y(-0{\cdot}70R + 1{\cdot}0G)$

Several determinations of the green point were made with a fairly satisfactory degree of agreement. The results are given in Table IV. Expressed in terms of a unit trichromatic equation, the mean value of the G′ stimulus is given by

$$G' = -2{\cdot}33R + 3{\cdot}33G.$$

A similar method to find B′, the blue stimulus, can be adopted, although the experimental possibilities are more limited. To obtain the maximum accuracy a test colour should be used as near to B′ as possible; 0·46 μ is the obvious choice. Using that as test colour and either a red or green adaptation, the colour becomes more saturated and cannot be matched. But with a blue adaptation the change is towards the centre of the triangle and the results can be measured. The experiments have therefore been limited to that condition, with 0·46 μ acting as the adapting light. The effects of this adaptation on 0·65 μ, 0·53 μ and 0·46 μ were measured and the calculations made on similar lines to those for the green response.

The results for one experiment are given in Table V; the intensity of the blue adaptation was about 70 photons.

Table V.

Wave-length and intensity of T.C.	R.	G.	B.	r_{ss}.	g_{ss}.	b_{ss}.
0·46 μ, 18 photons	—	—	35,320	100	40	2500
0·53 μ, 6 photons	—	619	—	80	200	—
0·65 μ, 3 photons	552	—	—	200	—	—

Now suppose B′ is given by

$$B' = xR + yG + zB.$$

After adaptation this will become

$$\frac{x}{552} \times 200R + \frac{y}{619}(80R + 200G) + \frac{Z}{35{,}320}(100R + 40G + 2500B)$$

$$= (0 \cdot 363x + 0 \cdot 129y + 0 \cdot 0028z)\,R + (0 \cdot 323y + 0 \cdot 0011z)\,G + 0 \cdot 0709zB.$$

For no colour change

$$\frac{y}{z} = \frac{0 \cdot 323y + 0 \cdot 0011z}{0 \cdot 0709z},$$

giving $y = -0 \cdot 0044z$.

Also

$$\frac{x}{z} = \frac{0 \cdot 363x + 0 \cdot 129y + 0 \cdot 0028z}{0 \cdot 0709z},$$

giving $x = -0 \cdot 0075z$.

Hence $B' = z\,(-0 \cdot 0075R - 0 \cdot 0044G + 1 \cdot 00B)$.

Two other similar experiments gave the following results,

$$B' = z\,(-0 \cdot 019R - 0 \cdot 0074G + 1 \cdot 00B)$$

and

$$B' = z\,(-0 \cdot 012R - 0 \cdot 0021G + 1 \cdot 00B),$$

with a mean value for the three experiments of

$$B' = z\,(-0 \cdot 013R - 0 \cdot 0046G + 1 \cdot 00B).$$

The unit equation for B′ is then

$$B' = -0 \cdot 013R - 0 \cdot 0047G + 1 \cdot 018B.$$

The agreement between the three experiments is on the whole satisfactory. The amounts of red and green involved are so small in comparison with the blue that a closer agreement could hardly be expected.

We can now collect the equations for our fundamental stimuli as follows :—

$$\begin{aligned} R' &= 1{\cdot}036R - 0{\cdot}036G, \\ G' &= -2{\cdot}33R + 3{\cdot}33G, \\ B' &= -0{\cdot}013R - 0{\cdot}0047G + 1{\cdot}018B. \end{aligned}$$

These equations can be combined with the relative luminosities of R, G and B and with the luminosity curve and trichromatic coefficients of the spectrum, for the author's eye, to calculate the fundamental response curves by well-known colorimetric transformations (Wright, 1929–30). These curves, for an equal-energy spectrum, are shown in fig. 8. In addition, in fig. 9 the spectral locus is shown in the colour triangle referred to R′, G′ and B′.

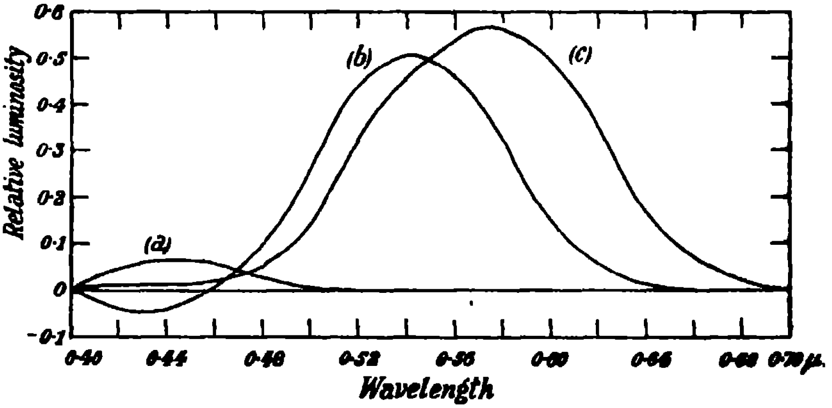

FIG. 8.—Fundamental response curves for an equal-energy spectrum, expressed in luminosity units. (*a*), blue ; (*b*) green ; (*c*) red.

A detailed discussion of these curves cannot be included in the present paper, but they would appear to provide a satisfactory basis on which to explain the other phenomena of colour vision, with the possible exception of the saturation differences of the spectral colours. The general characteristics of the curves are not very different from König's "fundamental sensation curves." The main difference is in the green curve at the shorter wave-lengths, where it is seen to go below the zero line at about 0·46 μ and become negative. The physiological mechanism by which such an effect could be produced cannot be visualized very readily, but it would apparently necessitate the assumption that all three fundamental responses have some quality in common, so that one response could produce a subtractive effect on another. This quality must probably be in the nature of an inherent "whiteness," and it is on an assumption of this sort that saturation differences might be explained.

On one point of previous dispute it is possible to come to a definite conclusion. It is quite certain that blue and not violet is the fundamental stimulus. It is only necessary to adapt the eye to a red radiation and observe the great change that is produced in a test colour at, say, 0·43 μ to prove that this radiation stimulates at least two responses.

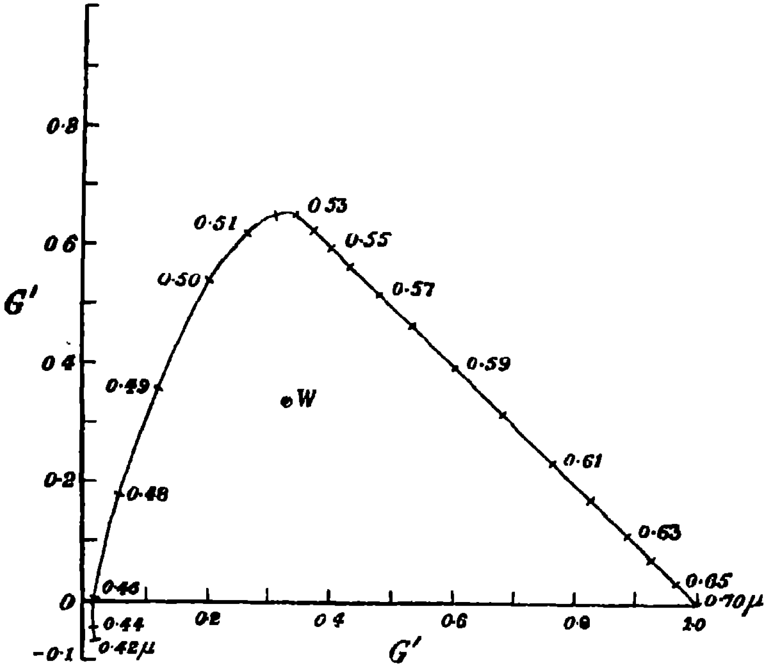

FIG. 9.—Locus of spectrum in colour triangle referred to fundamental stimuli, R', G', B'. W represents white point corresponding to black body radiation of 4800° K.

Variation of Intensity of Adaptation.

(*a*) ***Moderate Intensity Range.***—It is necessary to discuss the effect of varying the intensity of adaptation in two parts, under the headings of moderate intensities and high intensities. The phenomena in the two states differ to a marked extent, although there is, of course, a gradual transition from one state to the other. This transition occurs roughly within an intensity range between 10,000 and 20,000 photons, and in this section the adaptation phenomena occurring below the 10,000 photon level will be described.

In fig. 10 recovery curves are given for the red that were obtained in an experiment with a yellow test colour and a white adapting light of variable intensity. The intensity was controlled by rotating sectors with apertures ranging from 22½° to 180°.

The first point of interest is that, as with varying time of adaptation, the recovery curves are linear and with one exception, approximately parallel to one another. The recovery is somewhat slower with higher intensities of adaptation, but the change is comparatively small. This characteristic has been confirmed in a number of experiments; its significance is discussed below.

It is seen also that as the intensity of adaptation is increased, so the sensitivity decreases. The magnitude of the sensitivity at the end of the adaptation can be found by extrapolating the curves to $t = 0$. The values of r_0 can then be compared to the adaptation intensity A and their relation determined. This has been done in Table VI to obtain the very important result that the product Ar_0 is constant within small limits. (In the second adaptation, some disturbing factor has affected the observations, both as regards the slope of the curve and the value of r_0.) The experiment has been repeated a number of times and the results for three other series of observations are given in Table VII. Again, within quite close limits, the product is found to be constant. These results have been selected from a number of similar experiments, but although the values given here are the most consistent obtained, the others have only been inconsistent when the observations have been erratic, as shown by the scattering of the points, with occasional discrepancies similar to the second observation in Table VI.

Table VI.

Adaptation intensity (A) in photons.	r_0 (arbitrary units) [R = 3800; intensity of T.C. = 350 photons.]	Ar_0.
800	900	72×10^4
1330	420	56×10^4
2140	350	75×10^4
3200	250	80×10^4
4270	180	77×10^4
5340	140	75×10^4
6400	110	70×10^4

The intensity range that could be covered was limited, at high intensities, by the new phenomena that occur, and at low intensities by the need for the

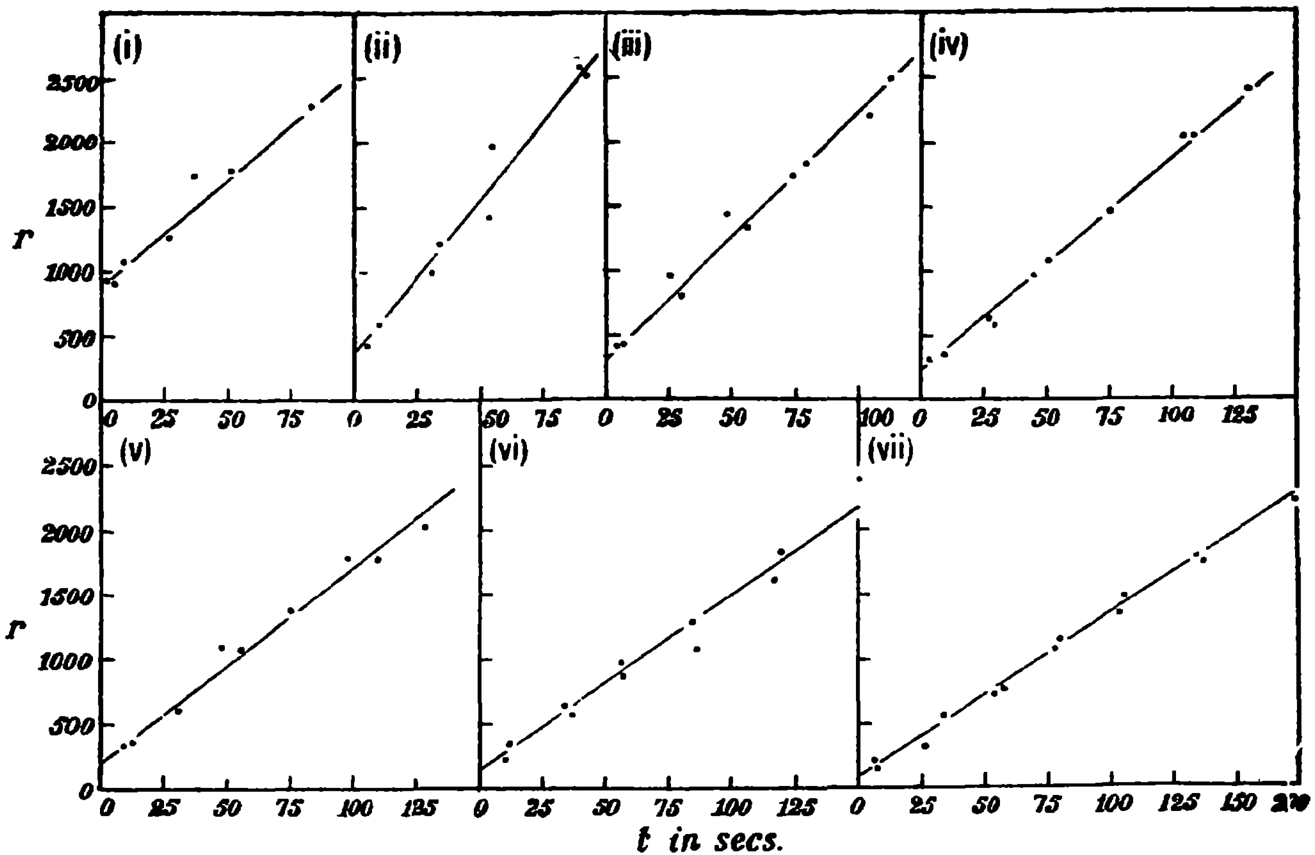

Fig. 10.—Red recovery curves for moderate intensities of adaptation, using white adaptation; intensities 800–6400 photons, and yellow test colour, intensity 350 photons, R = 3800. Adaptation intensity in photons: (i) 800, (ii) 1330, (iii) 2140, (iv) 3200, (v) 4270, (vi) 5340, (vii) 6400.

Table VII.

A. (photons).	g_e [G = 2130; T.C. = 320 photons]	Ag_e.	A. (photons).	r_e [R = 1175; T.C. = 110 photons.]	Ar_e.	A. (photons).	r_e [R = 361; T.C. = 300 photons.]	Ar_e.
1130	470	53×10^4	700	200	14×10^4	700	70	49×10^3
1870	290	54×10^4	1860	70	13×10^4	1210	40	48×10^3
3000	175	$52 \cdot 5 \times 10^4$	3720	40	15×10^4	2000	24	48×10^3
5000	120	60×10^4	5580	25	14×10^4	3200	16	51×10^3
8000	70	56×10^4						

test colour to be appreciably lower than the adaptation intensity. If a low intensity test colour were used, then after adaptation the apparent intensity would be much lower still and the matches would be very difficult to make with any degree of certainty.

Several very significant deductions can be made from the constancy of the product Ar_0. In the first place it is closely analogous to the observations associated with the Weber-Fechner law, in which two fields of intensity I are compared and one increased by ΔI until a just noticeable difference is produced. It has been found over intensity ranges similar to those used in the adaptation experiments, that ΔI/I is very nearly constant. Now if we assume that the sensitivity of the eye when adapted to intensity I is the same when the light is acting as it would be if the radiation were suddenly removed, then this constancy of ΔI/I exactly corresponds to the constancy of the products given in Tables VI and VII. The difference in the two derivations is that in one the stimulus required to produce a constant response (the just noticeable difference) is measured, while in the other the magnitude of the response from a constant stimulus (the fixed test colour) is determined.

Two deductions can be drawn from this agreement. First, the assumption that the sensitivity of the eye, when adapted, is the same when the light is entering the eye, as at the instant after it is cut off, evidently holds. This means that the sensitivity depends on the physiological condition of the eye and not on the physical stimulus producing that condition, a conclusion that has considerable logic of its own to support it.

Secondly, because ΔI is small in relation to I, it is taken as a direct measure of the sensitivity of the eye ; similarly because R, G and B are small in relation to the adapting intensity A, r, g, b can also be taken as direct measurements of the sensitivity, that is, of the magnitude of the visual response for a given stimulus. This should be noted as a further indication of the significance of r, g, b, as compared with $\log r$, $\log g$, $\log b$.

The implications of these conclusions can be further extended if we combine with them the effects described earlier when the intensity of the test colour was altered. It was shown that after adaptation with light of intensity A, the apparent intensity of a test colour would be reduced from R to r_0. From the coefficient law, a test colour of intensity 2R would be reduced to $2r_0$, 3R to $3r_0$ and so on.

In particular a test colour of the same intensity, A, as the adaptation would be reduced to a_0, where

$$a_0 = A \times r_0/R.$$

But it has been shown above that, for a constant value of R, $r_0 \times$ A is a constant product, hence

$$a_0 = A \times r_0/R = \text{constant}.$$

This equation implies that as the eye becomes adapted to any intensity A, the apparent brightness a_0 is gradually reduced to a constant level, no matter what the initial intensity A may happen to be. At first sight this is a very surprising conclusion. Actually it expresses algebraically the well-known fact that the eye adapts itself to approximately the same level over a wide range of intensities. The commonest illustration of this is the well-known phenomenon that a room artificially illuminated appears to be quite as well lighted, once the eye has adapted itself to that particular level, as it does in daylight, although the amount of light present is very much smaller.

The range of intensities over which the relation has been tested is comparatively small, but it is probable that the relation holds between the limits within which $\Delta I/I$ is approximately constant. Beyond these limits the deviation from constancy is an evident indication that the level of response cannot be maintained over the whole gamut of intensities. It will, in fact, be shown below that in all probability different adaptation mechanisms are brought into play at very high and very low intensities.

The mechanism by which a constant level of response is obtained can be easily visualized. Evidently the magnitude of the response must be determined by the rate of decomposition or disintegration of the photo-sensitive substance; when this decomposition is occurring at a steady rate the response will maintain a steady level. This would occur if the regeneration of the substance proceeded at a fixed rate, for under these circumstances when a stimulus acted, the amount of photo-sensitive material would either increase or decrease until the rate of decomposition was just neutralized by the rate of regeneration. A constant rate of regeneration would therefore involve, once the adaptation balance point had been reached, a constant rate of decomposition and thus a fixed level of response.

This conclusion is most satisfactorily confirmed by the linear nature of the recovery curves. Reasons have already been given for regarding r, g and b as direct measures of the response and the linearity of the recovery curves therefore implies a constant rate of regeneration of the light sensitive material. The fact that, in addition, the curves are approximately parallel for a wide range of adaptation intensity, provides a further elegant experimental confirmation of the theoretical deductions given above.

It has been shown that the instantaneous response to a stimulus A is reduced by adaptation in the ratio r_0/R to the fixed level a_0. The simple but interesting corollary follows at once that the initial response is proportional to

$$a_0 \times R/r_0 = a_0 \times A/a_0 = A,$$

the intensity of the physical stimulus. It would follow that if the Fechner fraction could be measured instantaneously, ΔI, instead of being proportional to I, would be constant. It would be very difficult to test this experimentally, but it is supported by phenomena such as "glimpsing," a property of the eye used by microscopists to detect faint details by glancing instantaneously at an object. On prolonged viewing the structure cannot be seen. This must not be confused with the opposite effect that occurs at very high intensities, when contrast is destroyed by the glare of the light, although when adapted the eye can readily detect differences in intensity that were previously invisible. Glare, however, produces abnormal processes in the eye that must be treated separately from normal conditions. They are discussed below.

Dark adaptation must also be treated as an abnormal condition. It is well known that the process of dark adaptation involves a special mechanism that takes a relatively long time, 20 or 30 minutes, to come into normal action, compared to the 2 or 3 minutes required for the eye to become light adapted, when passing, for instance, from a dark room to a lighted room or from daylight to an artificially lighted one. (The latter process, although occurring when going from a higher to a lower level of illumination, must still be considered a light adaptation process.) The slower dark adaptation process explains the prolonged recovery shown in fig. 4. The initial match is made when both left and right eyes are dark adapted; the recovery curve is obtained while the left eye is still dark adapted but while the right eye is recovering from light adaptation. The light adaptation process will recover rapidly, and will be completed after 3 or 4 minutes; but the return to the initial sensitivity will necessitate a further 30 minutes dark adaptation. As we are mainly concerned here with the processes of light adaptation, we have in general only measured the rapid recovery of the first 4 minutes. It must again be emphasized, however, that at the end of this period the remaining depression is very slight compared to that immediately following the adaptation.

This brief discussion does not explain the complete recovery of the blue. It will be more appropriately discussed when the effect of a variation in the wave-length of adaptation is examined.

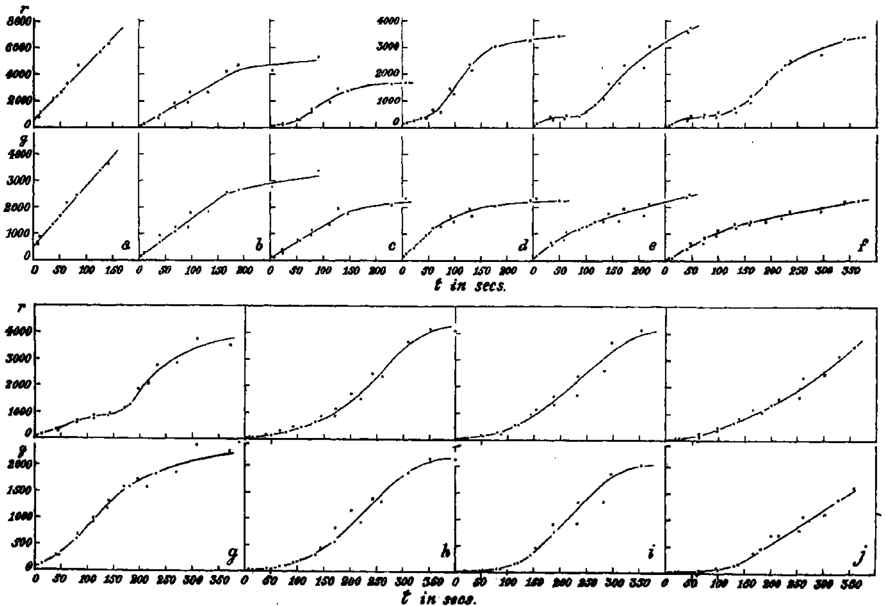

Fig. 11.—Recovery curves for high intensities of adaptation, using white adaptation; intensities 2500–92,000 photons, and yellow test colour, intensity 500 photons, R = 6900. G = 4100.

(*Note.*—Change of scale in r and g ordinates at higher intensities of adaptation.) Adaptation intensity in photons: *a* 2500, *b* 7100, *c* 9200, *d* 11,500, *e* 15,300, *f* 19,100, *g* 23,000, *h* 38,200, *i* 61,000, *j* 92,000.

(*b*) ***High Intensity Range.***—In fig. 11 the red and green recovery curves are shown for an experiment using a yellow test colour, intensity 500 photons, and a white adaptation varying in intensity from 2500 to 92,000 photons. Some of the curves that were obtained have been omitted, but those shown have been selected to illustrate successive stages in the high intensity recovery process.

It is seen that the linearity found with moderate intensities no longer holds; in some of the red curves the recovery occurs very slowly at first but after a considerable time, increases rapidly. At higher adaptation intensities, the recovery rate is fairly high at first, then slows down and almost stops and then increases rapidly again. Finally, at the highest intensity the recovery, while slow at first, follows a smooth curve throughout. In the green the irregularities are somewhat less marked, but the fact that the red and green behave differently is important as it may give a clue to understanding the mechanism that distinguishes the two responses.

It would appear that some change of state is taking place, such as the reformation of some substance or, more probably, the bleaching of an absorbing material. The latter possibility is supported by the fact that at these high intensities of adaptation, a trichromatic colour match made in the normal manner using only one eye, will no longer hold. For instance, if a yellow is matched against mixture of red and green, then after the eye has been adapted to a high intensity, the yellow will appear much redder than the mixture of the two primaries.

The recovery back to normal can be measured in a manner similar to that for the other recovery curves. The monocular match is made in the usual way, the eye is then adapted to the high intensity and at the end of the adaptation, the match is re-set. When the observations have been recorded, another match is made and so on until the recovery is complete. Results for an experiment on these lines is shown in fig. 12; a monochromatic yellow (intensity 300 photons) was matched with a mixture of the red and green instrument primaries to give

$$R = 2200, \quad G = 2200$$

before adaptation. After adaptation (white light, 18,000 photons) the green was unchanged, but the red had to be increased to obtain a match. The gradual return of the red to normal is shown in the diagram. The increase in the red is quite appreciable, being nearly double at $t = 0$. This suggests very strongly that the relative absorption of an intervening substance has been changed by the high intensity of light; either a transparent substance has become more absorbent in the red or a red substance has become more transparent in the yellow and green. Whether this takes place between H and P (of fig. 1) or at P itself is uncertain; it certainly cannot take place to the right of P, as it would not then produce a breakdown in the colour match.

There is little doubt that this effect is closely associated with the irregular recovery curves of fig. 11. More extensive observations, however, are necessary before a satisfactory analysis can be made of the visual reactions at these intensities and further deductions must be postponed until such observations are available.

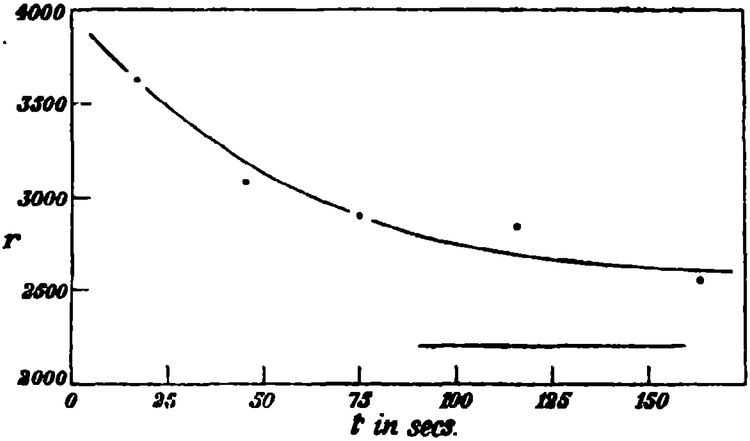

FIG. 12.—Breakdown of colour match; recovery of red to initial value of R = 2300 in monocular match between a yellow, wave-length 0·5825 μ, intensity 300 photons, and a mixture of red and green, after high intensity adaptation (white light 18,000 photons).

Variation of Wave-length of the Adapting Radiation.

In the initial stages of the work it was anticipated that it would be possible to analyse the changes in the relative depressions of the red, green and blue sensitivities as the wave-length of the adapting radiation was varied, and from the analysis deduce the fundamental response curves. Attempts to reach that goal in this way were unsuccessful, but a different line of attack has already been shown to have yielded the desired result. The failure of the first method was due to the fact that if, for example, one of the fundamental stimuli, R′, G′ or B′, could have been used as adapting radiation, then although the sensitivity of that response would be depressed most, yet all three responses would be depressed to a marked extent. For this reason the effects could not be analysed as a function of three variables and the method was eventually given up in favour of what proved to be a more direct approach to the problem.

It is impossible to describe the results of the innumerable combinations of test colour and adapting wave-length that could be investigated, and accordingly data are given relating only to a white test colour. These observations illustrate all the important features that have been encountered and more detailed results would tend to mask their outstanding characteristics.

In fig. 13 the recovery curves for the red, green and blue primaries are illustrated for a white test colour and adaptation radiations ranging from 0·65 μ to 0·43 μ. In fig. 14 these results, together with the result shown in fig. 3 for an adaptation with 0·56 μ, are plotted in the colour triangle to show the actual colour change produced by the adaptation.

Several significant features in these diagrams must be mentioned. With red adaptations the red sensitivity has, of course, been depressed most and with green adaptations the depression has been greatest in the green. Blue radiations, however, have not produced any preponderating blue depression and, as in other experiments, the blue has recovered most rapidly. It is important also to notice that with no adaptation is one response depressed excessively with respect to the others. (It should be remembered that if the recovery curves were measured in terms of the fundamental stimuli, R′, G′, B′, instead of R, G, B, the specific depressions would be still less evident.) For instance, with an adaptation of 0·65 μ it might reasonably have been anticipated that the green would hardly be depressed at all. The fact that it is very considerably depressed is an indication that there is a general, as well as a specific, adaptation mechanism. Further, it is very significant that with red adaptations, the green recovery is rapid compared with that when green adaptations are used, and with green adaptations the red recovery is more rapid. This is especially significant in view of the fact that when the intensity of adaptation is varied, only a small change in the slope of the recovery curves is produced.

Consider what happens when the light strikes the retina. Once the physical radiations have acted on the nerve endings, it is inconceivable that they should still maintain their individuality and independence. Hence the stage P, fig. 1, at which the responses are subdivided into three separate channels must inevitably occur at the same stage at which the radiant energy is converted into nervous energy; that is to say, the only action that can occur between H and P is one of absorption due to one or more coloured substances that may be interposed in the path of the light. The process at P is almost certainly responsible for the general adaptation phenomenon, in which some photosensitive substance is regenerated at a constant rate, and it follows that any

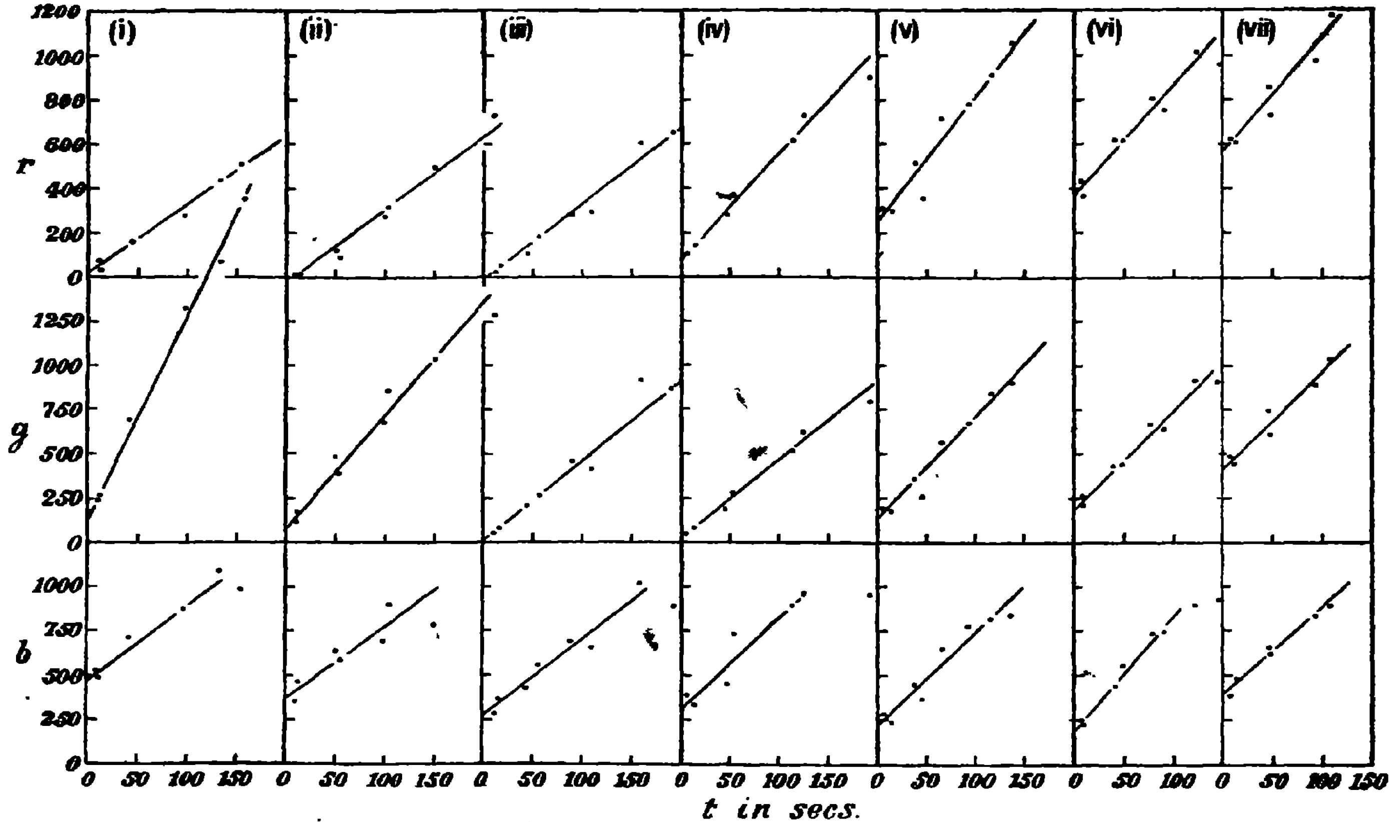

FIG. 13.—Recovery curves for various adaptation radiations; using white test colour, intensity 200 photons, R = 1750, G = 1900, B = 900.

	I	II	III	IV	V	VI	VII
Wave-length of adaptation, μ	0·65	0·60	0·58	0·53	0·49	0·47	0·43
Intensity of adaptation, photons	620	1920	2000	780	360	120	7·4

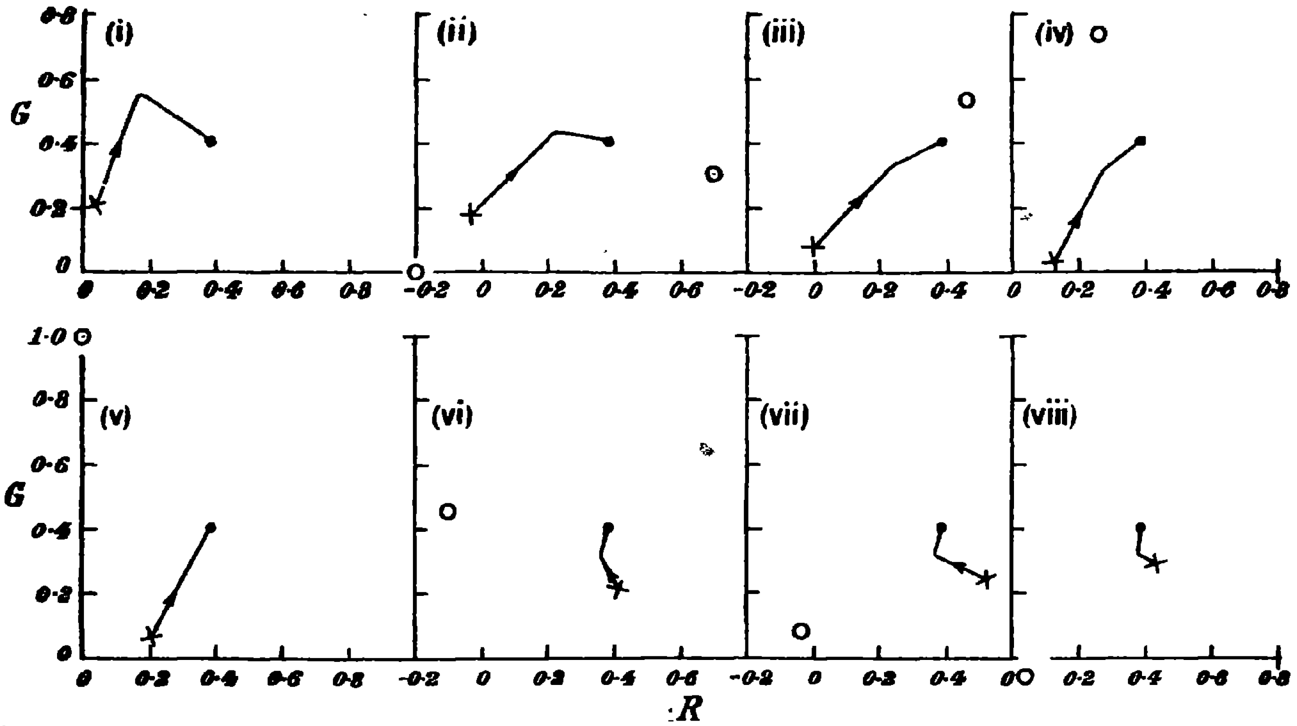

FIG. 14.—Colour change induced on white test colour, intensity 200 photons, trichromatic coefficients R = 0·384, G = 0·417, B = 0·199, by various adaptation radiations, as represented in colour triangle referred to instrument primaries, R, G, B. ○ Adaptation radiation ; ● white test colour ; × test colour after adaptation.

	I	II	III	IV	V	VI	VII	VIII
Wave-length of adaptation, μ	0·65	0·60	0·58	0·56	0·53	0·49	0·47	0·43
Intensity of adaptation, photons	620	1920	2000	1500	780	360	120	7·4

other process must be subsequent to this general process and will be composed of three specific effects occurring along each path, each effect being governed by the magnitude of the response along its own path.

We have seen earlier that usually the recovery is not completed for a relatively long time, as the enhanced sensitivity produced by the initial dark adaptation is a different and more prolonged process than that associated with light adaptation. Now it has just been shown that any process other than the light adaptation process is in all probability a specific effect and this will necessarily apply to the dark adaptation process. Further, the adapting radiation can only produce a specific effect along those paths in which it arouses a specific response and the effect of a radiation such as 0·65 μ will be to produce a general depression of sensitivity that affects all three responses, and a specific effect that is confined mainly to the R′ response. Hence the enhancement of the G′ response due to the initial dark adaptation will not be destroyed, thus permitting the rapid recovery of the green. Hence the change in slope of the curves as the wave-length of adaptation is varied is to be expected, while on the other hand variation of the intensity of a white adaptation, which produces responses along both the R′ and G′ paths, should cause only a small change in slope of the recovery curves.

If the same argument is applied to the blue curves, it would seem that no specific enhancement of the blue occurs during prolonged dark adaptation, since complete recovery from any period of light adaptation is always attained after 2 or 3 minutes. This abnormal behaviour of the blue may also be associated with the absence of any specific blue depression with blue adaptations and with the small positive blue observed when red, yellow or green test colours are used.

It must, of course, be realized that this discussion of dark adaptation refers only to the fovea and cone vision. While the processes may be similar in extra-foveal vision, no conclusions arrived at in this paper can be legitimately extended to the rods.

The diagrams in fig. 14, while of general interest, are of no special theoretical value in themselves. The change in direction of the recovery paths, for instance, is simply the result of one response recovering before the others. It is interesting to note that only with adaptation radiations in the region of 0·58 μ, is the induced colour change in the direction of the complementary colour.

Previous Investigations.

There have been many researches carried out on adaptation by other investigators, but while many of them have been of a quantitative nature, a large number have merely been descriptive and of no permanent value. A number of investigations have been directed towards the development of the trichromatic theory, but although numerical data have been obtained, the results have almost universally suffered from two main defects. In the first place, the measured quantities have only been indirectly related to the colorimetric characteristics of vision and no very useful interpretation of the results has been possible. In the second place, in measuring the effects of adaptation, no account has been taken of the recovery that sets in immediately the adapting light is cut off. Thus it has been the common practice to give results without stating the time from the end of the adaptation at which they were recorded; and no attempt has been made to measure the actual course of the recovery process.

For these reasons it is impossible to make any very valuable comparisons between the present work and that of other experimenters. So far as they go, the results of Hess (1890), Burch (1897), Edridge-Green (1921), Porter (1912), Abney (1913), and Hamilton and Laurens (1923) are in agreement with the results given in this paper. Abney's experiments are perhaps the most closely allied to the present work, but they are not exempt from the two defects mentioned above.

Allen (1932) has made a series of experiments in which he determined the sensitivity of the eye before and after adaptation by measuring the critical frequency of flicker for various test colours. His experiments certainly suffered from the second defect in that no record is given of the time at which the observations were made, nor is the course of recovery determined. His conclusions have been criticized by Guild and the author (1932) on the grounds that flicker phenomena found for one state of adaptation cannot safely be extended to other conditions of adaptation. The chief erroneous conclusion is that adaptations with some radiations enhance the sensitivity of the eye to other wave-lengths. Sufficient evidence has been brought forward in this paper to show that when the eye is adapted to a given intensity A, its sensitivity is less than when it is adapted to a smaller intensity αA, α being less than unity. Allen's results may be due, not only to the uncertain nature of the flicker experiment, but also to the uncertain relation between the intensities of his adaptation radiation and test colour and the level of light adaptation in which the eye was initially maintained, none of which are precisely defined.

Lythgoe and Tansley (1929) also using the critical frequency of flicker method, have investigated the sensitivity of the eye under different conditions of adaptation, and although their results do not lend themselves to any direct comparison with those given in this paper, the two sets of results are not contradictory at any point.

Numerous experiments on the Weber-Fechner law have a bearing on the subject of this paper, but they cannot be referred to here in detail. An experiment by Schjelderup (1920), however, is of great value in supporting the conclusion that the instantaneous response is proportional to I and not to log I. He carried out experiments using a test field of intensity i and a surround field of intensity I and found that Weber's formula was modified to

$$\Delta i = ai + bI,$$

a and b being constants. When I was appreciably higher than i, Δi became independent of i. This is exactly what should occur on the basis of the argument developed earlier. The high surround intensity produces a state of adaptation that is unaffected by the lower intensity of the test field ; a change in the magnitude of the test stimulus thus produces a change in the response without any change in the state of adaptation. The experiment effectively eliminates adaptation as a variable, and under these circumstances it is found that to produce a given change in the response, a constant increase in the stimulus is required and not an increase proportional to i.

Measurements by Chaffee and Hampson (1924) on the action potential of a frog's retina showed that previous stimulation with monochromatic radiations did not produce any specific effect on the action potential developed by other radiations, but caused a more or less uniform depression of sensitivity throughout the spectrum. This is in agreement with the conclusion arrived at above, that the major effect is a general depression of sensitivity.

Hecht (1929) has elaborated an attractive photo-chemical theory of adaptation, but the particular equations postulated do not agree with the experimental data obtained here. The paucity of useful information on which to base a theory has meant that all such attempts must inevitably be very precarious and uncertain. The response curves also deduced by Hecht are not supported by the colour adaptation experiments. They have also been criticized on other grounds.

In addition to the papers already referred to, very valuable surveys of the subject and reviews of previous work on this and allied phenomena are given by Parsons (1924), Lythgoe (1926) and Adams (1929).

Conclusions.

So many theories of visual processes have been built up in the past, the majority on very insecure foundations, that further attempts can only be justified when new information of a significant character is forthcoming. Even then, the reasoning linking physical measurement with its physiological counterpart must always be accepted with caution, and measurements on colour adaptation cannot be excepted from this rule. For this reason it is not proposed to amplify the deductions that have already been made, but merely to summarize them briefly.

It has been shown, then, that there is almost certainly both a general and a specific adaptation mechanism, and it has been found that the general process operates by the existence of a photo-sensitive substance that is regenerated at a constant rate. The results also show that the instantaneous response aroused by a stimulus is directly proportional to the magnitude of the latter (over a mean range of intensity), but owing to the process of adaptation, the response is rapidly reduced to an approximately constant level. The effect of varying the wave-length of adaptation is to produce a greater relative depression of sensitivity of one or other of the three responses, but the results seem to suggest that the incident light reacts with one photo-sensitive substance to produce three different products, rather than with three distinct photo-sensitive substances. The proportions in which each radiation stimulates the three responses have been determined, to give the fundamental response or excitation curves. Experiments with high intensities of adaptation have shown that either the photo-sensitive material is broken down in some abnormal manner or that the spectral transmission of an intervening pigment or substance is altered by the high intensity.

These results are not sufficient in themselves to determine the unique system that underlies the phenomena, but they do provide a framework that should be of material value in eventually forming a satisfactory theory of colour vision. It is to be hoped that direct physiological evidence will be forthcoming to confirm the conclusions arrived at here and to amplify and extend them.

In conclusion the author wishes to express his very sincere thanks to Mr. W. J. Morgan, who assisted in the initial stages of the work; to Mr. F. H. G. Pitt, who had the rather thankless task of recording the majority of the observations and rendered very valuable help in many other ways; to Professor L. C. Martin for his interest and suggestions throughout the work; and to the

Medical Research Council for their financial assistance and permission to publish these results.

Summary.

The effects of light adaptation have been measured by first matching the test colour, as viewed by the right eye, against a mixture of three primaries derived from a trichromatic colorimeter, as viewed by the left eye. The right eye then gazes at a third and more intense patch of light until it is adapted to the higher intensity level, when the test colour is viewed again. The effect of the adaptation is measured by re-matching the primaries against the test colour and successive matches give the course of recovery of the initial state of adaptation. The left eye thus acts as a standard of reference against which the changes produced by various conditions of adaptation have been measured; for different intensities and colour and for varying times of adaptation; and for various wave-lengths and intensities of test colour. The analysis of the results shows that there is a main process of adaptation that operates through the regeneration of a photo-sensitive substance at a constant rate. The instantaneous response aroused by a stimulus is found to be directly proportional to the magnitude of the latter, but owing to the process of adaptation the response is rapidly reduced to an approximately constant level. This is shown to be the true interpretation of the constancy of the Fechner fraction, as opposed to the suggestion that the response is proportional to the logarithm of the stimulus. By locating the three hypothetical stimuli in the colour triangle corresponding to those sensations that can be modified in intensity but not in colour, no matter what the colour of the adaptation may be, it has been possible to determine the fundamental response or excitation curves. The curves obtained do not differ very greatly from König's "fundamental sensation curves." Measurements on the effect of varying the wave-length of adaptation show that there is a specific effect on each response as well as a general depression of sensitivity, and the results indicate the probability that the incident light reacts with one photo-sensitive substance to produce three different products, rather than with three distinct photo-sensitive substances. Experiments with high intensities of adaptation show that with retinal illuminations above about 15,000 photons, one or more of the visual processes break down and abnormal effects are introduced.

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Experimental Pituitary Basophilism.

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[PLATES 3–5.]

The dual purposes of this study were: (1) to determine whether prolonged injections of the pituitary gonadotropic hormone would reproduce in animals any semblance of the malady known as "pituitary basophilism"; and (2) thereby to throw further light on whether the basophilic elements of the pituitary body are actually the source of this hormone, as has long been assumed though without wholly conclusive proof.

Briefly stated, clinical gigantism and acromegaly are associated with adenomas composed of acidophilic cells of the pars distalis and these disorders may therefore be regarded as states produced by "pituitary acidophilism." Another more recently described clinical syndrome (Cushing, 1932) is associated with an actively secreting adenoma composed of basophilic elements, this condition in contrast having been conveniently termed "pituitary basophilism."

The conception that these and other adenomas of the ductless glands such as those of the parathyroid glandules and pancreatic islets represent actively secreting conglomerations of normal cells rather than inert pathological lesions has only of late come to be fully realized. The syndromes produced by the pituitary adenomas differ from all others because of the widespread polyglandular changes which they evoke. The only satisfactory way to study the nature and extent of these secondary glandular effects and thus to expedite our knowledge of the pituitary disorders is by reproducing them in animals.

After many years' delay the experimental reproduction of gigantism and acromegaly was finally brought about (Evans and Long, 1921; Putnam, Benedict, and Teel, 1929; Evans, Meyer, and Simpson, 1933) by long-continued injection of the growth hormone. The gonadotropic principle, on the other hand, has hitherto been employed only for its acute sex-maturing effects. Apart from the experimental production of gigantism and acromegaly and the recent prolan-plus-growth-hormone experiments by Evans and his co-workers (1933, *b*), no reports of long-term injections in animals of other principles of the hypophysis have been made.

Gonadotropic Hormones.—We are not directly concerned here with sex-maturing substances other than those obtained from extracts of the pituitary body. When in 1927 Aschheim and Zondek announced their discovery in the urine of pregnant women of the substance "prolan," it was thought that a new and convenient source of the pituitary sex hormone had been found. Evidence, however, from many sources (*e.g.*, Engle, 1929, 1933; Evans and Simpson, 1929, 1932; Riddle and Polhemus, 1931; Wallen-Lawrence and Van Dyke, 1931; Leonard, 1933; and Shockaert, 1933) serves to make clear that the two substances while similar in their action are not identical.

Nevertheless, that prolan bears some close relationship to pituitary function is unquestioned and Evans and his associates have recently demonstrated that when combined with growth extract it is rendered far more active than otherwise. Injected into rats and dogs (Evans and co-workers, 1933, *b*) this mixture produces external evidence of œstrus and lactation, and internal evidence of activity of the organs of reproduction. So far as we can see, these interesting observations have no bearing on the matter in hand.

Methods of extracting the gonadotropic principle from the pituitary body have been given by Bugbee, Simond, and Grimes (1931), by Van Dyke and Wallen-Lawrence (1933), by Fevold, Hisaw, and Leonard (1931), and in a publication which appeared just as our experiments were concluded, Evans, Meyer, and Simpson (1933, *a*, pp. 87 *et seq.*) have described what is probably the most effective method for its preparation. While no comparative study has been made of the precise effects of these different extracts, there can be no doubt that a highly active, water-soluble principle, which causes stimulation of the gonads of immature and hypophysectomized rats, may, by all three methods, be obtained from pituitary glands, more abundantly from those of sheep.

I. *Preparation of the Extract.*

The different methods of preparing an extract which were available to us were: (*a*) the alkaline tricresol method of Bugbee and co-workers (1931), (*b*) the pyridine method of Fevold and co-workers (1931), and (*c*) the alcohol precipitation method of Van Dyke and Wallen-Lawrence (1933). For our purposes the alcohol method was found, after prolonged trials, to be most suitable and guided by the authors' description of the procedure, with a few modifications to suit our facilities and convenience, the extract was prepared as follows.

One hundred fresh sheep pituitary glands, cleaned of bone and dural fragments, are well ground in a mortar with two or three volumes of powdered quartz, and this mixture is suspended in 1 litre of 0·02/N ammonium hydroxide. This suspension is allowed to extract with frequent mixings for 24 hours at room temperature. Enough N/1 hydrochloric acid is then added to bring the p_H of the mixture to approximately 5·6, and when equilibrium is reached the mixture is centrifuged.

Five volumes* of 95% ethanol are then added to the clear, pinkish, supernatant fluid, and after stirring, the precipitate is allowed to settle-out during the night so that in the morning most of the supernatant fluid may readily be removed by suction. The remaining sludge is centrifuged, the supernatant discarded, and the precipitate taken up in distilled water. A large portion of the precipitate remains insoluble, and these irreversible proteins are removed by centrifugation.

The supernatant fluid, containing the active principle, is again precipitated with five volumes of 95% ethanol, and from this point the extract is treated as if sterile. The centrifugation of the alcoholic precipitate is made in sterile cups, and the precipitate is resuspended, this time in 500 c.c. of sterile Ringer's solution. It is again centrifuged, to remove as much as possible of the remaining irreversible proteins, and is finally placed in a sterile bottle, capped with a rubber stopper through which a needle can be introduced for removal of small quantities.

The final product thus represents derivatives of one gland for each 5 c.c. of extract. Further extraction of discarded precipitates did not yield enough activity to warrant pursuit. Cultures were taken on broth of four different batches of the extract, and one culture was made for anaerobes; all were negative.

In brief, the extract as prepared above was presumed to contain nothing but the gonadotropic hormone of the pars anterior. The growth hormone and lactation principles were supposedly removed in the insoluble alcohol precipitate, and the posterior lobe hormone was either destroyed by the alkali or carried away in the supernatant alcoholic solution.†

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* Five volumes of alcohol rather than exactly 70%, as called for by Van Dyke *et al.*, are used because this has been the concentration used by Smith and Smith (1933) in their extractions of the blood and urine of eclamptics. Since we were already familiar with other phenomena of this type of extract, it was thought best to make comparable preparations. Also, extracts of urine of pregnancy made by the same method were compared with the hypophysial extract.

† There was a possibility that minute quantities of posterior lobe extract might have been carried down into the precipitate. However, 65 c.c. of the extract injected intravenously in a dog produced no alteration of pulse, blood-pressure, or respiration. Ninety

II. *The Effect of the Extract upon Rats.*

(*a*) *The Hundred-hour Test on Immature Rats.*—When tested on immature female rats by the Zondek-Aschheim method, a cubic centimetre of the preparation was found to contain from one to two rat units, depending upon the batch tested. The reaction produced was luteinization of the ovaries with no appreciable effect upon the uterus, which remained pale and small. The vaginal membrane did not rupture.

(*b*) *The Hundred-hour Test on Hypophysectomized Rats.*—Young, female rats which had been hypophysectomized before attaining sexual maturity and which had been observed for several weeks were first subjected to laparotomy for examination of the ovaries and uterus. From two animals whose ovaries showed no corpora lutea, one ovary of each was excised for histological study. After recovery from the operation each of the animals was injected with four rat units of the extract divided into six doses. One hundred hours later the animals were killed and when examined each of the remaining ovaries showed several small corpora lutea, with definite interstitial activity.

(*c*) *Long-term Injections into Rats.*—Immature female rats fed on an effective growth-stimulating diet* were given daily injections for 65 days with 2 c.c. of the extract. This dose, depending upon the potency of the particular batch, represented two to four rat units per day. The animals were weighed frequently, and at intervals vaginal smears were made for several days in succession.

The first abnormal effect to be noted was the development of pronounced raggedness of the animals' fur which became coarse and brittle. If an area were shaved, the regrowth of hair was slow and sparse. Although the injected animals became quite fat, in two to three weeks it was noted that the gain in weight to be expected of rats on the special diet did not take place. In spite of their good appetite and normal health, the injected rats fell behind their uninjected littermate sisters in weight and even more in stature.

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minutes after the injection the blood sugar was 163 mg. %. The dog had been fasting for at least 18 hours. Dr. Oliver Kamm, of Parke, Davis and Company, kindly tested the extract for us and found it free from both pressor and oxytocic fractions. He happened at the same time to test the preparation on the pigeon and found that in addition to its sex-maturing response it gave the reaction of prolactin. This effect would not have been observed on our immature puppies.

* This diet was devised by Osborne and Mendel (1926). The growth produced by this and other diets when fed to normal and hypophysectomized rats has been reported in detail (Thompson and Gaiser, 1932).

The rupture of the membrane of the introitus was much delayed in the injected rats. When opened, both introitus and vagina appeared atrophic. Vaginal smears of all of the injected animals showed a few epithelial cells and many leucocytes but never a smear typical of oestrus. In some instances male rats were allowed to remain for weeks in the cages with the injected females, but no pregnancy resulted. A definite temperamental change in the animals shown by irritability with their fellows was also observed.

The most marked pathological variations from normal were noted in the excessive amount of body fat, atrophy of the thymus and hyperplasia of the ovaries. No appreciable change, either gross or microscopic, was observed in the adrenal, parathyroid, or thyroid glands. The ovaries were markedly overdeveloped and numerous corpora lutea were present. The uterus remained small and atrophic.

III. *The Effect of the Extract on an Immature Dog.*

The experimental animal and its littermate sister were pedigreed, white fox-terrier females 8 weeks old. Both were well formed, active and healthy, and almost identical in their appearance. The animals lived separately in metabolism cages, but were taken out of doors for exercise two or three times daily. Once a day they were generously fed an identical diet which was modified from time to time as they grew older. Once every week cod liver oil was added to their food. During the 90-day period of observation neither dog suffered from any illness.

How well a puppy would tolerate subcutaneous injections of the extract was unknown at the beginning of the experiment, nor had it yet been determined whether the extract had been rendered wholy free from posterior lobe hormone, the necrosing effects of which might conceivably be troublesome. For these reasons the initial dose was small. Starting with 1 c.c. it was increased to 50 c.c. in the course of the first week as tolerance to the subcutaneous injection was demonstrated.

Pathological Findings.—No new features having developed both animals were killed on September 8, the 93rd day of the experiment, by the intravenous injection of 80 c.c. of air. The examinations of the bodies were carried on simultaneously by two observers. The contrast between the normally lean control and the adipose experimental animal was even more apparent after death. The vulva of the injected animal was surrounded by clusters of fat, suggesting the appearance of a scrotum, but there were no external evidences of sexual maturity.

Protocol.

Date.	Control animal.	Injected animal.
June 5	Weight: 3210 grams. Urine: negative.	Weight, 3370 grams. Urine: negative.
June 7	—	Daily subcutaneous injections of extract begun.
June 13	Weight: 3600 grams. Fasting blood sugar: 113 mg. %.	Weight: 3630 grams. Fasting blood sugar: 114 mg. %.
June 14	—	50 c.c. extract, daily to August 1.
June 28	Photographs taken.	No difference in animals noted.
July 1	—	Beginning adiposity first observed.
July 10	Weight: 4800 grams.	Weight: 5250 grams. Obesity and retardation of growth noticeable.
July 12	Total of 10-day urine output: 2375 c.c. Examination negative.	Total of 10-day output: 3890 c.c. Examination negative.
July 14	Photographs taken.	Marked difference in animals apparent, figs. 1, 2, Plate 3.
July 15	Weight: 5000 grams.	Weight: 6080 grams.
July 18	R.B.C.: 6,050,000. Hb: 90%. Blood serum calcium: 10·4 mg. % (fasting). Plasma phosphatase: 0·32 units.	R.B.C.: 4,700,000. Hb: 70%. Blood serum calcium: 12·4 mg. %. Plasma phosphatase: 0·36 units. Sugar tolerance test (5 mg. sucrose per kg.): Fasting …… 103 mg. %. ½ hour …… 124 ,, 1 ,, …… 129 ,, 2 ,, …… 144 ,, 3 ,, …… 140 ,,
	Basal metabolism readings made.	Metabolism 70% below that of control. Five readings were taken, 4 were consistent.
July 26	Serum calcium: 11·5 mg. % (fasting).	Serum calcium: 12·2 mg. % (fasting). Noticeable retardation in dentition. X-rays of long bones show no appreciable structural change.
August 1	Weight: 5460 grams. Length: 69·8 cm. Area on the right flank shaved.	Weight: 5860 grams. Length: 61·1 cm. Same, for study of growth of hair. Daily dose of extract increased to 75 c.c. until August 20.
August 8	Sugar tolerance test (5 gm. sucrose per kg.): Fasting …… 102 mg. %. 1 hour …… 138 ,, 2 ,, …… 115 ,, 3 ,, …… 110 ,,	Sugar tolerance test (5 gm. sucrose per kg.): Fasting …… 98 mg. %. 1 hour …… 144 ,, 2 ,, …… 135 ,, 3 ,, …… 120 ,,
August 11	Photographs taken. Shaven area covered by new hair, fig. 3, Plate 3.	Shaved area remains hairless and dusky in colour. Animal playful and affectionate with keepers, but cross with and jealous of her control. Protuberance of abdomen obviously increasing.
August 16	Photograph to show retarded dentition of control, figs. 4, 5, Plate 4.	A loose eyetooth extracted for histological study showed nothing definite.
August 17	Serum calcium: 13·6 mg. % (fasting). Serum phosphorus: 7·5 mg. % (fasting). (Serum badly hemolised.)	Serum calcium: 14·3 mg. % (fasting). Serum phosphorus: 5·2 mg. % (fasting). (Slightly hemolised.)

Protocol—(continued).

Date.	Control animal.	Injected animal.
August 18	Weight : 5800 grams. Urine : negative.	Weight : 6300 grams. Urine : negative.
August 19	Metabolism determinations.	Readings average 59% below littermate sister. Four readings : all consistent.
August 20	—	Daily dose of extract increased to 100 c.c.
August 21	Length : 69·7 cm. (tip of nose to tip of tail).	Length : 59·3 cm.
August 23	R.B.C. : 6,600,000. Hb : 90%. W.B.C. : 14,900. Differential : Polymorps 72% Lymphocytes 28% Eosinophils 0% Basophils 0% No abnormal cells	R.B.C. : 4,480,000. Hb : 70% W B.C. : 16,000. Differential : Polymorphs 63% Lymphocytes 27% Monocytes . 6% Eosinophils 3% Basophils 1% No abnormal cells.
August 24	Total urinary output in 10 days : 1700 c.c : negative.	Same : 1040 c.c. : negative
August 25	Serum calcium . 11·9% mg. % (fasting). Serum phosphorus . 6·0 mg. % (fasting).	Serum calcium : 13·1 mg. % (fasting). Serum phosphorus : 6·3 mg. % (fasting).
August 29	Sugar tolerance test (5 gm. of sucrose per kg.) : Fasting 128 mg. %. 1 hour 154 ,, 2 ,, 128 ,, 3 ,, 118 ,,	Sugar tolerance test (5 gm. of sucrose per kg) : Fasting ... 96 mg. % 1 hour 132 ,, 2 ,, 179 ,, 3 ,, 185 ,,
August 30	No glycosuria to 200 gm. of sucrose.	Glycosuria marked to 200 gm. of sucrose.
August 31	Photographs. Shaved area obliterated by new hair, fig. 6, Plate 4.	Shaved area few coarse hairs. General accentuation of former condition. Folds of fat encircle body (*cf.* figs. 6, 7, 8, Plates 4, 5). Personality change more marked.
September 2	No glycosuria to 200 gm. of sucrose. Arterial blood pressure (femoral puncture) 178/170.	Slight glycosuria to 200 gm. of sucrose. Arterial blood pressure (femoral puncture) 170/165.
September 6	Aschheim-Zondek urine test : negative. (Rabbits and rats ; two observers.)	Same tests also negative.
September 7	Urinary output in past 10 days : 1700 c.c.	Same : 1030 c.c.
September 8	Weight : 5900 grams. Arterial pressure (femoral cannula) 160/120.	Weight : 6400 grams. Arterial pressure (femoral cannula) 140/110.

The most striking difference on section was the quite unexpected pallor of the tissues of the injected animal. While this was more marked in the muscles it involved practically all structures except the enlarged and prominent liver. The change was even apparent in some of the ductless glands which were carefully removed, dissected clean of connective tissue and immediately

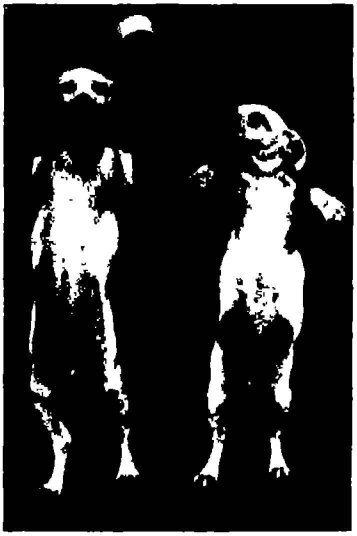

FIG. 1.

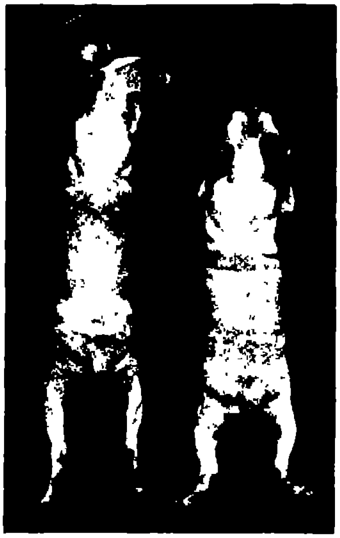

FIG. 2.

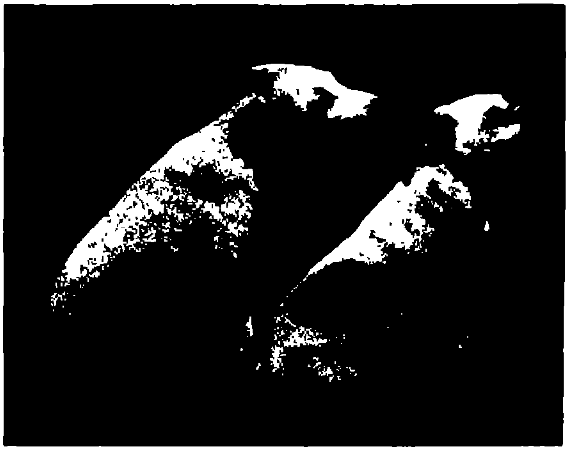

FIG. 3.

FIGS. 1 and 2.—Comparative condition of animals on July 14 after 37 days; test animal on right.

FIG. 3.—Comparative appearance of shaved areas 10 days later.

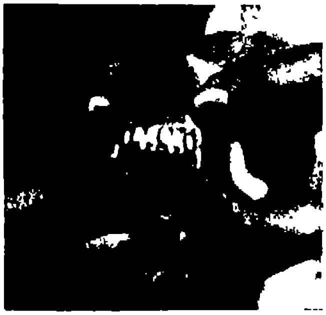

FIG. 4.

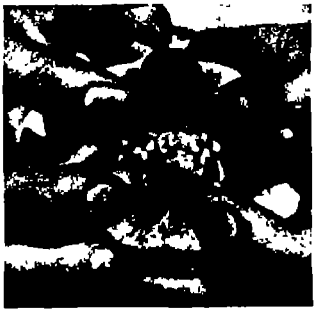

FIG. 5.

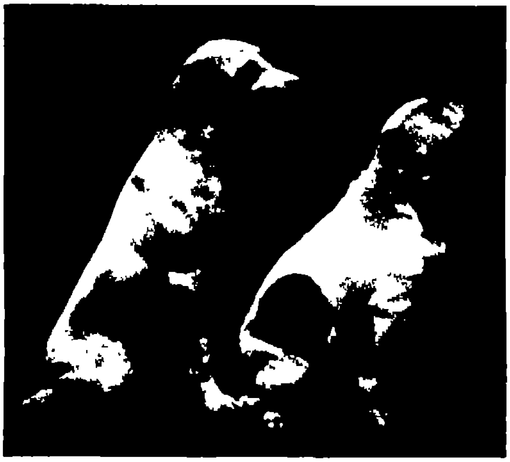

FIG. 6.

FIGS. 4 and 5.—The comparative state of dentition of control and injected animals on August 16th, after 72 days.

FIG. 6.—Comparative appearance of shaved areas in the two animals 30 days later.

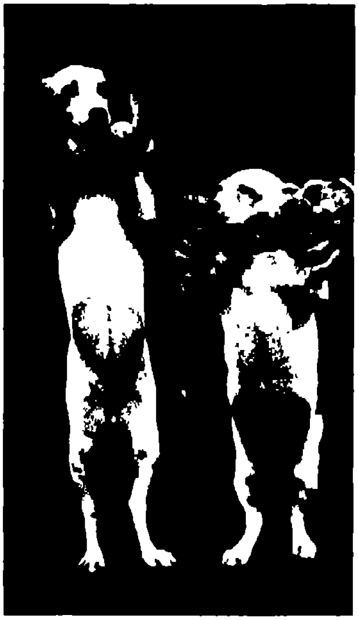

Fig. 7

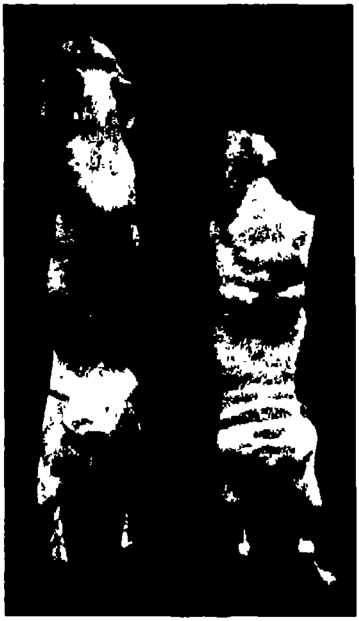

Fig. 8

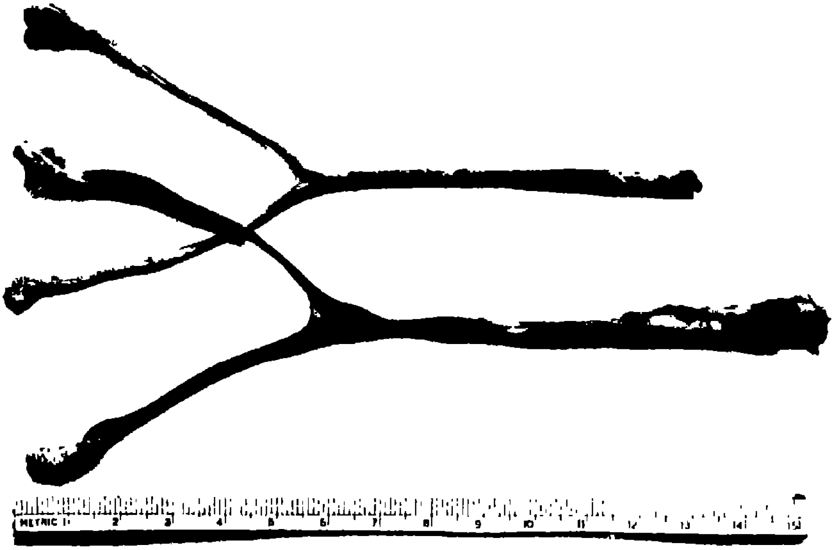

Fig. 9.

Figs. 7 and 8. Comparative condition of animals on August 31st, after 85 days of continuous injection of the test subject to right.

Fig. 9.—The uteri and ovaries of the two animals (control below).

weighed, Table I. Apart from this colour change there was no observable gross difference in the size or appearance of the endocrine organs. The *uterus* and *ovaries*, however, were atrophic, pale and small, the entire reproductive system being less well developed than the still immature sex organs of the control, fig. 9, Plate 5.

The abdomen and pelvis of the injected animal contained great masses of fat, and the mesentery was exceptionally fatty. The *heart* was smaller than that of the control; the *liver* was one-third larger and the stomach slightly larger. The *spleen*, on the other hand, was only half the normal size and the *kidneys* were somewhat smaller, the cortex measuring 1 mm. thinner than the control.

The *bones* gave evidence of lack of development. Nodes at the costochondral junctions showed on cross-section a U-shaped line of ossification, as contrasted to the straight line of the normal. The femur of the injected dog was 9·3 cm. long compared with the normal, 12·2 cm. The tibia of the injected dog was 9·7 cm., the normal, 12·9 cm. The vertebræ were much smaller in the injected dog. X-ray studies showed no discernible changes of a pathological nature either in the long bones or skull of the injected animal.

Table I.—Weights of Organs.

	Normal, gm.	Control, gm.
Thyroid gland	1·01	1·00
Thymus gland	3·37	3·69
Heart	60·0	50·0
Liver	370·0	490·0
Stomach	80·0	95·0
Pancreas	17·1	17·6
Spleen	11·5	6·53
Uterus and ovaries	2·11	0·98
Kidneys	76·8	59·8
Adrenals	0·72	0·78

Histological Examination.—The histological deviations from the normal in the tissues of the injected animal are chiefly seen in the thyroid, uterus, ovaries, muscle, and liver. There are no changes anywhere apparent in the peripheral arterioles. The *spleen*, *pancreas*, and *pituitary glands* of the two animals are indistinguishable. The convoluted tubules of the kidney of the injected animal show a considerable amount of fat in the cells, which is not apparent in the control.

The *thyroid* of the test animal shows large acini distended with inspissated, heavily staining colloid and lined by exceedingly low epithelium. This is in marked contrast to the normally active appearing gland of the control in which the acini are small, the amount of colloid scanty, and the epithelium of high cuboidal type. The *parathyroid glandules* of the test animal show no recognizable evidence of activation. They contain a greater number of cells of large type, but mitotic figures were detected only in the glandules of the control. The slightly larger *adrenals* show the cells in the zona fasciculata to be larger than those of the control owing to increase of lipoid.

The *uterus* compared to the controls shows an infantile type of endometrium. The *ovaries* are distinctly less advanced in their activity than those of the control in which animal there are many maturing follicles with abundant granulosa and multiple ova.

In the enlarged *liver* of the test animal the histological change is most striking. There is no deposition of fat as was expected, but the cells are highly distended with glycogen. Professor Wolbach states that he has encountered a similar change only in von Gierke's disease.

A remarkable change was also apparent in the test animal's *striated muscle*, both cardiac and peripheral. Whereas in the muscle of the control animal the striations are sharply shown, in the test animal they are scarcely distinguishable. Unfortunately no pieces of muscle were fixed in alcohol so that staining for glycogen was precluded.

Summary of Laboratory, Clinical, and Pathological Notes.

From her littermate sister, who during the 93 days of observation grew into a lanky, active animal, the injected puppy differed in the following ways :—

(1) She had become exceedingly adipose, slow in movement and petulant in disposition. She nevertheless appeared to be in good health.

(2) There was a retardation of skeletal growth, delay in the normal dentition, and extremely slow growth of hair. The serum calcium values were invariably higher than in the control. Calcium elimination, however, was not determined and the bones showed no roentgenological or pathological evidence of decalcification. The parathyroid glandules showed no demonstrable hyperplasia.

(3) Compared with the control, she had a markedly subnormal basal metabolic rate and the thyroid proves to be definitely colloidal in type with low epithelium.

(4) The carbohydrate tolerance was diminished though there was no spontaneous glycosuria. The liver (and possibly the muscle) was heavily loaded with glycogen.

(5) No nephro-vascular disturbances were clinically or pathologically evident. There were no hypertensive effects, rather the reverse, the blood-pressure having been lower than in the control. The blood showed a moderate anæmia and the spleen was small. A striking pallor of most of the tissues was apparent after death.

(6) Instead of the expected sexual ripening, the normal maturation was retarded compared with the control, the uterus and ovaries of the injected puppy being the more infantile of the two at the conclusion of the experiment.

Discussion.

Both in experimental gigantism of rats, as first produced by Evans and Long (1921) and in the experimental canine acromegaly produced by Putnam, Benedict, and Teel (1929), the growth extract employed was contaminated by some of the gonadotropic principle whose separable effects were not yet fully appreciated. The experimental syndrome was consequently a mixed one in which unaccountable secondary changes in the reproductive organs occurred. Evans and his co-workers (1933, *a*) using a more purified extract have since produced overgrowth in dogs without disturbing the organs of reproduction, though the other secondary polygrandular effects such as those on the thyroid and adrenal glands together with an hypophysio-pancreatic-islet imbalance all occurred.

Thus canine acromegaly has been experimentally produced without co-incidental disturbance of the apparatus of sex. In this disorder as it occurs in man the gonadal functions are not impaired by the excess of growth hormone but only when the adenoma producing it reaches a sufficient size to inhibit by pressure the elaboration of the gonadotropic hormone. When secondary gonadal effects do occur, therefore, they are always those of inactivation as expressed by amenorrhœa and impotence. This at least is our interpretation of the malady.

While the growth hormone has thus come definitely to be associated with the acidophilic elements, there has been doubt expressed regarding the cellular source of the anterior hypophysial gonadotropic substance. It has been thought to be a product of the basophilic elements, but this has never been satisfactorily proved. It has seemed reasonable to assume that if some of the more striking clinical features of pituitary basophilism could be produced in animals by the long-term injection of a gonadotropic extract of the pituitary body it would tend to establish this relationship.

Pituitary basophilism occurs more frequently in women than men, and more often in young persons, particularly at or about the time of adolescence. How early it may occur is unknown for there have been no cases in which the disorder has been pathologically verified in early childhood. Its syndrome will doubtless be found to differ at different ages, but its general features in young persons are a rapidly acquired adiposity; a retardation of growth; a peculiar dusky plethora of the skin; falling of the hair of the scalp while the secondary hirsutism of sex becomes exaggerated; a partial decalcification of the skeleton with increased calcium elimination; a tendency toward hyperglycæmia; inactivity of the thyroid shown by a low basal metabolic rate; amenorrhœa with atrophy of the uterus; and finally, hypertension with terminal atherosclerosis.

Like acromegaly, pituitary basophilism therefore is a polyglandular disorder in which the activity of parathyroids and adrenals appears to be increased and of thyroid and pancreatic islets diminished. The sex glands (supposedly stimulated at the outset) ultimately become inactivated so that in the female the ovulatory cycle is not completed and the uterus atrophies.

In this experiment with two littermate, pedigree fox terriers, a condition highly suggestive of the clinical disorder was produced. The injected animal became adipose and plethoric; the body growth was definitely checked; the growth of teeth and hair was retarded; the basal metabolic rate was markedly subnormal; the serum calcium was high and the sugar tolerance low.

Wholly missing from the syndrome, on the other hand, were the expected early maturation,* the erythremia, and the evidences of hypertension. On the contrary, the ovaries and uterus remained small and failed to show in comparison with the control even their normal development. The blood changes, if anything, were on the side of a destructive process rather than the reverse, with a smaller spleen, a lower blood count and hæmoglobin than the control, together with a lower blood pressure. At autopsy a peculiar pallor of the muscles as well as of other tissues such as the thyroid was striking.

The massive glycogen storage in the liver and its possible relation to von Gierke's disease, in which a similar condition occurs, would appear particularly significant. In this disorder the pituitary body does not appear to have been examined nor has an hypophysio-pancreatic-islet imbalance been suggested as its cause. The normal glycogenic content of the hepatic cells is greatly diminished both in hyperinsulinism and in hypopituitary states. Conversely, it is increased both in islet insufficiency and in hyperpituitarism whether of acidophilic or basophilic types.

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* The absence of any ovarian activation, comparable to the Aschheim-Zondek reaction, need perhaps not be surprising. In the single recorded example (Bishop and Close, 1932) of verified pituitary basophilism that had started in pre-adolescence at the age of 11, the uterus and ovaries at the time of the patient's death when 22 years of age were infantile in type.

Diabetes mellitus, in other words, may be produced either by islet insufficiency or by pituitary overactivity, some even believing in the existence of a separable diabetogenic hormone in the hypophysis. The carbohydate-metabolizing balance between pancreas and hypophysis is such that, as Houssay (1931) has shown, after an hypophysectomy the pancreas may be removed without ensuing diabetes. A small amount of insulin given to an animal under these experimental circumstances quickly leads to fatal hypoglycæmia unless the effect is counteracted by the simultaneous injection of a sufficient amount of pituitary extract.

Conclusions.—Whether this inaugural experiment had better have been made on an animal more nearly adolescent, whether it should have been far more prolonged in order to show the full effects of the extract, or whether a more potent or more highly purified preparation should have been employed are questions only to be answered by further study.

As the experiment stands, with all its limitations, it strongly suggests the experimental reproduction of certain features of the clinical syndrome of pituitary basophilism. And as this disorder is known to be associated with, and probably to be caused by, an unmixed adenoma of purely basophilic elements it may safely be concluded that the gonadotropic extract employed in the experiment (shown by the Aschheim-Zondek test to be active) was a product of the basophilic cells of the sheep's glands from which the preparation was made.

It required forty years and more to reproduce in animals Marie's acromegaly so that it could be properly analysed and studied. It will certainly not take so long before pituitary basophilism is reproduced, and this study, it is hoped, will prove to be a preliminary step in that direction.

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Action of X-Rays on the Eggs of Calliphora.

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[Plate 6.]

Introduction.

The object of the experiments described in this paper was to investigate the factors which determine the differences shown by cells in regard to their sensitivity to the lethal action of X-rays when different types of cells are compared. This sensitivity is found to vary over an enormous range. For example, Crowther (1926) showed that a dose of 80,000 r administered in 20 minutes was required to kill the protozoon *Colpidium colpoda* while, on the other hand, the author (1933) found that under special circumstances a dose of 2–3 r administered over a period of 2 hours could kill the cells of the allantois of the embryo chick.

The information concerning the sensitivity of cells to short wave radiations has been summarized in the so-called Law of Bergonie and Tribondeau (1906) which states that the sensitivity of a cell is proportional to its reproductive capacity and inversely proportional to its morphological and functional differentiation. Mottram (1913) amplified the information summarized in this law by conclusions derived from experiments on growing root tips of *Vicia Faba* and on developing *Ascaris* ova. He concluded that cells were most sensitive to irradiation during the actual process of nuclear division.

In the investigation described in this paper the biological effect of X-rays was estimated quantitatively by measuring the percentage of eggs of *Calliphora erythrocephala* (the common bluebottle) which were prevented from hatching by any given dose of X-rays. In general the method is similar to that of Packard (1926–1931) who used the eggs of *Drosophila melanogaster* for a similar purpose. In my experiments I attempted to correlate the changes in sensitivity to irradation which were found to occur in the developing calliphorine egg with the changes in the morphological structure of the egg.

Experimental Methods.

The object of these experiments was to obtain curves relating the amount of exposure to X-rays with the action produced on batches of eggs under a variety of experimental conditions. Accurate measurements can only be obtained if the eggs are as uniform and as healthy as possible and the precautions necessary to obtain this result are detailed below.

(*a*) *Method used for Breeding* Calliphora erythrocephala.—A constant stock of adult flies was kept in order to provide eggs. All the flies were the offspring of a single wild fly and about 20 generations of flies were used in these experiments. The flies were kept in two disused fume cupboards at a temperature of about 15° C. They were fed on loaf sugar and were provided with water. Eggs were obtained by exposing fresh uncooked liver to the flies which laid exclusively on this medium. When a new stock was required the liver and the eggs that it bore were incubated at 23° C. In about 17 hours larvæ hatched and began to feed on the liver. The larvæ and the liver on which they were feeding were placed in a biscuit tin, the bottom of which was covered with 2 to 3 inches of dry silver sand. The larvæ were incubated at 23° C. in the tin, and after becoming sluggish they migrated into the sand on the fifth day from hatching. Here they pupated, and hatching occurred after a further period of 12 to 13 days of incubation at 23° C. Immediately before the hatch the biscuit tin was transferred to an empty fume cupboard. Fresh liver was provided as food for the newly hatched flies, but as soon as they began to lay eggs, which they did after 2 or 3 days, the liver was removed and loaf sugar was substituted. Each batch of stock flies numbered about 1000. The adult flies lived for several months at 15° C., but after the first month a large proportion of the eggs which they laid were infertile. For this reason the stocks of flies were renewed every month.

(*b*) *Method used for Collecting and Manipulating the Eggs.*—Eggs were obtained for experimental purposes by exposing a piece of fresh liver to the flies. About a thousand eggs per hour were laid and this rate was maintained for about 4 hours, provided that the flies were allowed to lay only on alternate days. Each piece of liver was exposed for half an hour. The eggs that were laid during the first hour were discarded because at the beginning of a laying period, eggs which were in an advanced state of development, and even larvæ, were often deposited. Apart from these exceptions, caused by the retention of fertilized eggs in the oviduct, a half-hour batch of eggs was of almost uniform development because the calliphorine egg is fertilized immediately before laying.

For experimental purposes eggs were removed from the liver by means of a platinum loop and were separated from each other in order to allow counting. They were plated on to a piece of moist blotting paper 4 cm. by 1 cm. which was stuck on to an ordinary microscope slide. The slide was contained in a Petri dish in which the air was saturated with water vapour. From a half-hour laying about eight slides, each with about 60 eggs, were prepared and the eggs were counted. Four slides were irradiated and four were used as controls. After irradiation the experimental eggs and the unirradiated controls were incubated at a known thermostatically controlled temperature. When the hatch of the control eggs was complete the unhatched eggs were counted on the control and on the experimental slides.

(*c*) The source of X-rays was a Müller, water-cooled, hot cathode tube with a tungsten anticathode. In all experiments the kilovoltage, milliamperage, and distance from anticathode to target were constant. These were 79 k.v., 5 m.A., and 47·1 cm. A screen of aluminium 0·01 cm. thick was used in all experiments. The output of the tube was measured regularly by means of a gold leaf electroscope of the usual pattern and was kept constant by small alterations of milliamperage. The dose of X-rays delivered at 47·1 cm. was approximately 40 r. per minute.

During irradiation the microscope slides on which the eggs had been plated were contained in a box which was kept at a constant temperature, fig. 1. The box was made of lead and the atmosphere inside it was saturated with water vapour by means of wet filter paper attached to the walls. The slides were contained in a lead rack which accommodated three slides for irradiation and three control slides.

(*d*) *Measurement of the Effect of Irradiation.*—The percentage of eggs which were prevented from hatching by the irradiation was the measure of the action of the irradiation. Extensive control experiments were therefore necessary in order to find the percentage of eggs which hatched normally. The control eggs were approximately equal in number to the irradiated eggs and, except for irradiation, were treated in exactly the same manner. Over 20,000 control eggs were used and 92% hatched. The percentage hatch was very uniform and experiments were discarded in which the percentage hatch of the control eggs was less than 90. Histological examination of unhatched control eggs never revealed any embryological development nor even the presence of a nucleus and it was therefore concluded that the unhatched control eggs, or at least the majority of them, had escaped fertilization. In expressing the percentage of eggs which had been prevented from hatching by irradiation a correction for unfertilized eggs was necessary and this was made on the basis of the corresponding control experiment.

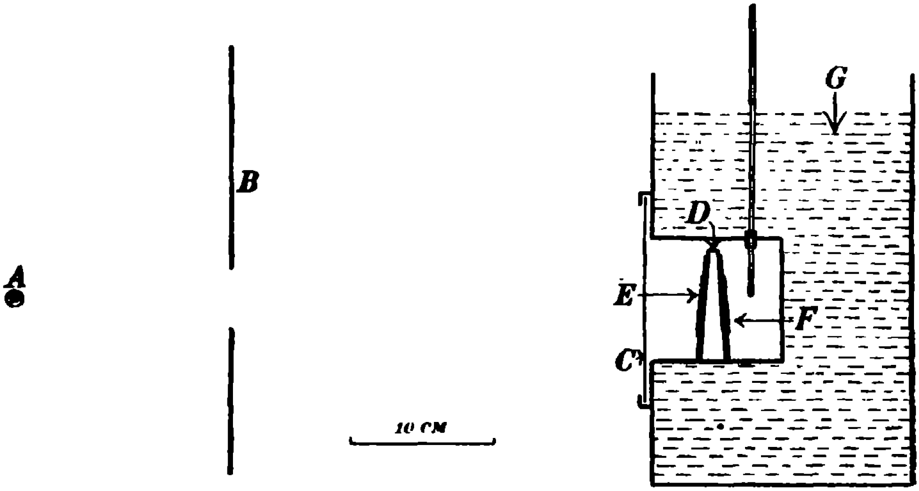

FIG. 1.—Arrangement of apparatus. A, anticathode; B, lead screen; C, aluminium screen 0·01 cm. thick; D, lead rack for holding slides; E, irradiated eggs; F, control eggs; G, water bath.

Influence of Temperature on Rate of Development.

Eggs were incubated at different temperatures and the times elapsing between deposition and hatching of the eggs were measured. The following results were obtained: at 8° C., 120 hours; at 13° C., 62 hours; at 17° C., 34 hours; at 23° C., 17 hours.

These results, which are plotted in fig. 2, show an almost exact linear relationship between the temperature and the logarithm of the time. The Q_{10} calculated from the graph is 3·64 and this appears to be constant within the limits of experimental error over the range of temperatures investigated. This temperature coefficient is similar to that found by other observers for the development of insects (Uvarov, 1931).

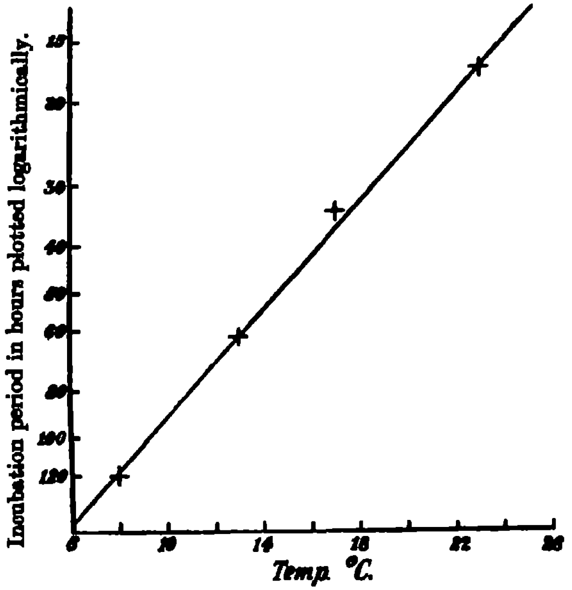

FIG. 2.—Relation between the incubation period and temperature (Q_{10} = 3·64).

The high figure for the Q_{10} made it essential to control the temperature rigidly in order to investigate the relationship between the stage of development of the egg and its sensitivity to X-rays. The uniformity of the Q_{10} between 8° C. and 23° C. made it possible to express the duration of incubation of an egg at a given temperature within the range considered in terms of the duration of incubation at some other temperature, which would result in a similar stage of development. This conclusion was confirmed later by means of the histological examination of the eggs. This method was sometimes used for convenience to avoid prolonged incubation at low temperatures which would often have involved work throughout the night. In this paper all the durations of incubation are expressed in terms of incubation at 14° C.

Effect of X-irradiation upon Eggs at Various Stages of Development.

The effects produced by irradiation of eggs at various ages are shown in Table I and in figs. 3–5 and are summarized in fig. 6.

Fig. 6 shows that the changes in sensitivity are complex. There is a very great decrease in sensitivity between 4 and 7 hours incubation which is preceded and followed by a moderate increase in sensitivity. After about 16 hours the sensitivity decreases steadily.

Table I.

Dose.		Eggs incubated for 0·3–1 hour at 14° C.				
In r units.	In minutes.	No. of experiments.	No. of fertile eggs calculated from control experiments.	Average of the percentage hatch in individual experiments.	χ^2.	P_{χ^2}.
20	0·5	1	27	96·4	—	—
40	1·0	5	277	83·0	16·9	<0·01
60	1·5	2	117	81·4	0·2	0·7–0·5
80	2·0	8	431	67·5	16·4	0·05–0·02
100	2·5	6	434	61·7	50·8	<0·01
120	3·0	4	255	46·2	31·5	<0·01
160	4·0	3	174	18·5	4·2	0·2–0·1
200	5·0	3	169	15·7	7·9	0·02–0·01
240	6·0	3	231	9·1	1·9	0·5–0·3
280	7·0	1	55	1·8	—	—
320	8·0	1	74	0	—	—

Dose.		Eggs incubated for 1–2 hours at 14° C.				
In r units.	In minutes.	No. of experiments.	No. of fertile eggs calculated from control experiments.	Average of the percentage hatch in individual experiments.	t.	P_t.
20	0·5	1	40	100	—	—
40	1·0	8	472	81·9	0·11	0·9
60	1·5	2	84	68·7	1·20	0·3–0·4
80	2·0	7	339	63·2	0·63	0·5
100	2·5	3	143	42·4	1·49	0·2–0·1
120	3·0	7	316	38·8	0·89	0·4–0·3
160	4·0	8	356	23·5	0·47	0·7–0·6
200	5·0	5	229	5·7	0·96	0·4–0·3
240	6·0	6	264	2·6	2·95	0·05–0·02
280	7·0	7	450	0·3	—	—
320	8·0	1	130	0	—	—

t and P_t compare the averages for eggs of 1–2 hours incubation with the averages of eggs of 0·3–1 hour incubation.

Table I—(continued).

Dose.		Eggs incubated for 2·5–3·5 hours at 14° C.				
In r units.	In minutes.	No. of experiments.	No. of fertile eggs calculated from control experiments.	Average of the percentage hatch in individual experiments.	t'.	$P_{t'}$.
20	0·5	1	89	79·8	—	—
40	1·0	5	378	63·6	1·95	0·1–0·05
60	1·5	2	173	40·3	1·71	0·2–0·1
80	2·0	5	342	12·0	10·30	$<$0·01
120	3·0	6	435	5·2	8·00	$<$0·01
160	4·0	5	349	0·7	—	—
200	5·0	3	220	0	—	—
240	6·0	2	137	0	—	—
280	7·0	2	134	0	—	—
320	8·0	1	58	0	—	—

t' and $P_{t'}$ compare the averages for eggs of 2·5–3·5 hours incubation with the averages of eggs of 1–2 hours incubation.

These complex changes can be most conveniently considered in three stages :—

(*a*) The changes in the first 3·5 hours.

(*b*) the changes after 10 hours.

(*c*) The changes between 3·5 and 10 hours.

(*a*) *Action of X-rays on Eggs less than* 3·5 *hours old.*—Experiments were made on eggs at the following ages :—

I. Eggs between 0·3 and 1 hour. The results are recorded in Table I and fig. 3, curve A.

II. Eggs between 1 and 2 hours. The results are recorded in Table I and fig. 3, curve A.

III. Eggs between 2·5 and 3·5 hours. The results are recorded in Table I and fig. 3, curve B.

In the three groups of experiments eggs which had been laid during a half-hour period were plated and were incubated at 14° C. for 20, 70, and from 120 to 180 minutes respectively. They were irradiated and then incubated along with the corresponding control eggs at 23° C. until the hatch occurred. The percentage hatch was then measured. The total number of control eggs used in this series of experiments was 5350 and of these 4921 or 92% hatched. The eggs were obtained from about 50 different layings and 10 different stocks of flies were used.

The results show that the average sensitivity of the eggs to X-rays does not vary significantly during the first 2 hours of incubation at 14° C., but that after 2·5 hours the eggs become more sensitive. The dose of X-rays required to kill 50% of eggs of less than 2 hours incubation is 100 r, while only 50 r produce the same effect in eggs which have been incubated for 2·5–3·5 hours.

Table I and fig. 3 show, however, averages obtained from many experiments, and, in order to decide whether the differences observed are significant, it is necessary to consider the scatter of the individual experiments. Fig. 4 shows the individual results in typical samples of experiments carried out on eggs less than 2 hours old and more than 11 hours old. The variation shown in fig. 4 is so great that a statistical analysis is necessary. The method of analysis is described below and the summary of it is expressed in Table I in columns headed χ^2, P_{χ^2}, t, and P_t.

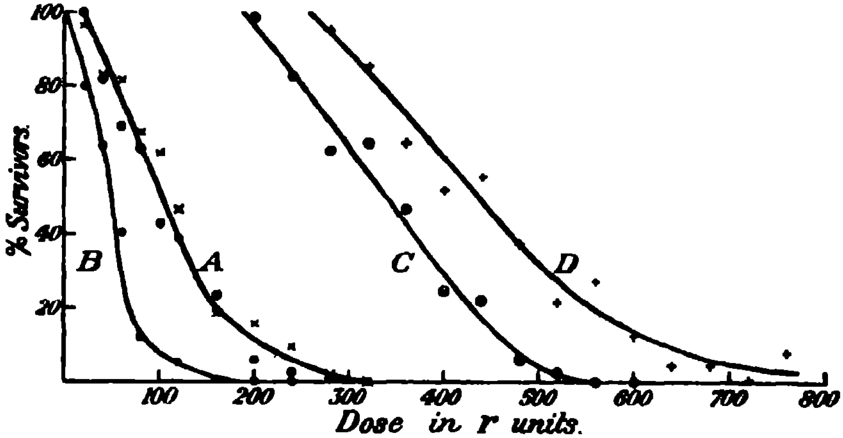

FIG. 3.—Action of X-rays on eggs incubated for times equivalent at 14° to—Curve A, ×, 0·3–1 hour; ●, 1·0–2 hours; curve B, ○, 2·5–3·5 hours; curve C, ⊙, 11 hours; curve D, +, 16 hours.

This analysis showed that the variation in the results was not due to the random sampling of the eggs and, therefore, in expressing the mean percentage of eggs which were killed by a given dose of X-rays, the following method was adopted. Several different batches of eggs were exposed to the same dose of X-rays and the percentage effect in each batch was measured. These percentages were averaged and this figure was considered as the mean percentage of eggs killed by a given dose of X-rays rather than the percentage derived from the total number of eggs used and the total number of eggs killed.

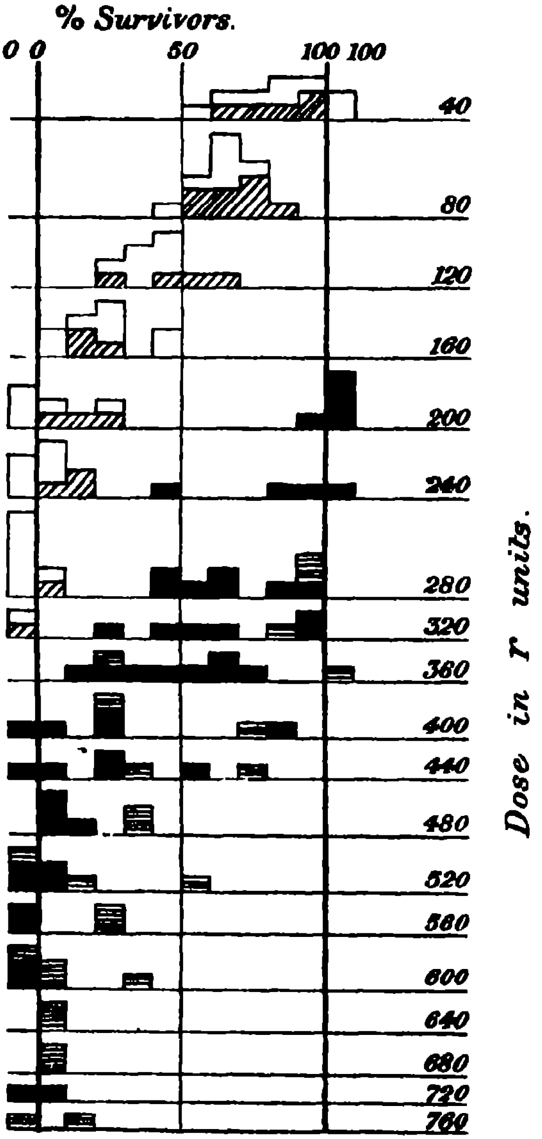

FIG. 4.—Distribution graph showing the scatter of results of typical experiments. Eggs incubated for times equivalent at 14° C. to: 0·3–1 hour, ▭ ; 1–2 hours, ▨ ; 11 hours, ■ ; 16 hours, ▤.

Analysis of Results.

The detailed results which are summarized in Table I were examined in order to find whether the variation which they show was due to random sampling of the eggs. The analysis was made by the use of the statistic χ^2 (Fisher, 1928, p. 69). By means of an appropriate statistical table (Fisher, 1928, p. 96) the value of χ^2 reveals the probability that the variability is due to random sampling. This probability has been recorded in Table I in column headed P_{χ^2}. For example, if $P_{\chi^2} = 0 \cdot 5$ then if the variation of the results is due to a sampling error there are 5 chances in 10 that an equal or greater value for χ^2 will be obtained. If $P_{\chi^2} = 0 \cdot 01$ there is only 1 chance in 100 that χ^2 will be equal to or greater than the value obtained; in other words, if $P_{\chi^2} = 0 \cdot 01$ the variation of the numbers is probably not due to a sampling error. Table I shows that the variations in my results are almost certainly not due to sampling errors and the simpest explanation of the variations is found in the rapid changes of sensitivity of the eggs which are illustrated in fig. 6. Averages of results were therefore calculated, not from the total number of eggs used, but from the individual percentage action obtained from each group of eggs.

The averages recorded in Table I were compared with each other by means of the statistic *t* (Fisher, 1928, p. 107). By means of an appropriate table (Fisher, 1928, p. 139) the probability of the significance of the differences between averages may be estimated. This probability has been expressed in Table I under the headings P_t and $P_{t'}$. For example, if $P_t = 0 \cdot 5$ for two series of results, and if the two series are in truth part of the same series, then the difference between the averages which occurred experimentally or a greater difference will happen 5 times out of 10 by chance. If $P_t = 0 \cdot 01$ the difference in the averages will occur only once in a hundred times by chance.

In other words, the smaller the value is for P_t, the greater is the probability that there is a significant difference between the averages. The values recorded for P_t show that for a given dose of X-rays there is no significant difference between the averages recorded for eggs of 0·3–1 hour incubation and the averages for eggs of 1–2 hours incubation. $P_{t'}$, however, shows that the difference between the averages for eggs of 1–2 hours incubation and the averages for eggs of 2·5–3·5 hours incubation is a significant difference.

(*b*) ***Action of X-rays on Eggs of more than* 10 *hours Incubation*.—**

(i) ***Eggs incubated for* 11 *hours*.**—The results are recorded in figs. 3 and 4.

(ii) ***Eggs incubated for* 16 *hours*.**—The results are recorded in figs. 3 and 4.

(iii) *Eggs incubated for* 22 *hours.*—The median lethal dose of these eggs was found to be considerably greater than 1200 r. units. This dose killed about 10% in one or two experiments, but in the majority of experiments all the fertile eggs hatched.

(iv) *Eggs incubated for more than* 22 *hours.*—Eggs incubated from 22 hours up to hatching which occurs at 54 hours were so resistant that it was not possible to estimate the median lethal dose. The hatched maggot was also so resistant that no apparent effect was produced by doses up to 2500 r.

In all these types of experiment the development of the egg was accelerated for experimental convenience, the method being as follows. The eggs were laid during a half-hour period and were incubated at room temperature (14° C.) for 1 hour. They were then incubated at 23° C. for various times and this duration of incubation was converted into terms of incubation at 14° C. by the use of the factor Q_{10}, fig. 2. This figure shows that a rise in temperature from 13° C. to 23° C. caused a 3·64-fold increase in the rate of development and that this increase was approximately uniform. A rise of temperature from 14° C. to 23° C. would, therefore, cause a 3·19-fold increase in the rate of development or in other words incubation for 1 hour at 23° C. is equivalent to incubation for 3·19 hours at 14° C.

After irradiation the eggs in all four series of experiments were incubated at 23° C. until hatching occurred.

The results of experiments on the action of X-rays on eggs of more than 10 hours incubation at 14° C. are summarized in fig. 6. This figure shows that the sensitivity to X-rays decreases enormously between 16 and 22 hours of incubation. The sensitivity decreases slightly between 11 and 16 hours, but at 22 hours the median lethal dose is more than four times the median lethal dose at 11 hours.

(*c*) *Action of X-rays on Eggs Incubated for Periods of between* 3·5 *and* 10 *hours.*—Experiments were made on eggs at the following ages in hours: 3·5–4, 4–4·5, 4·5–5, 5–6, 6·5–7, 8·5–9. The results obtained during these periods showed a much greater scatter than those obtained with eggs at other ages. The results of the individual experiments are shown in fig. 5. It will be seen that in some cases the mortality produced by a given dose of X-rays on batches of eggs of the same age varied from 0 to 100% and a comparison of fig. 5 with fig. 4 shows the striking difference in variation inside and outside the limits of from 3·5 to 10 hours.

Averages obtained from the figures for eggs between 3·5 and 10 hours would have a doubtful significance and therefore these experimental results have not been averaged in tabular form as was done with the remainder of the experiments. The points in fig. 5 resemble the results obtained at other periods in that each point represents an experiment made on from 60–100 eggs. Fig. 5 shows that the majority of the results obtained with eggs between 3½ and 4 hours old lie close to curve B which is that obtained with eggs between 2½ and 3½ hours old, but a few of the experiments show a greatly increased resistance. The results with eggs from 4 to 5 hours old show a wide scatter, but all lie between the curves B and C. Curve C is for eggs 11 hours old, from fig. 3. The results with eggs from 5 to 6 hours old are scattered around curve C, whilst all except two of the results with eggs from 6½ to 7 hours old lie to the right of curve C. Finally the results obtained with eggs 8½ to 9 hours old are scattered around curve C.

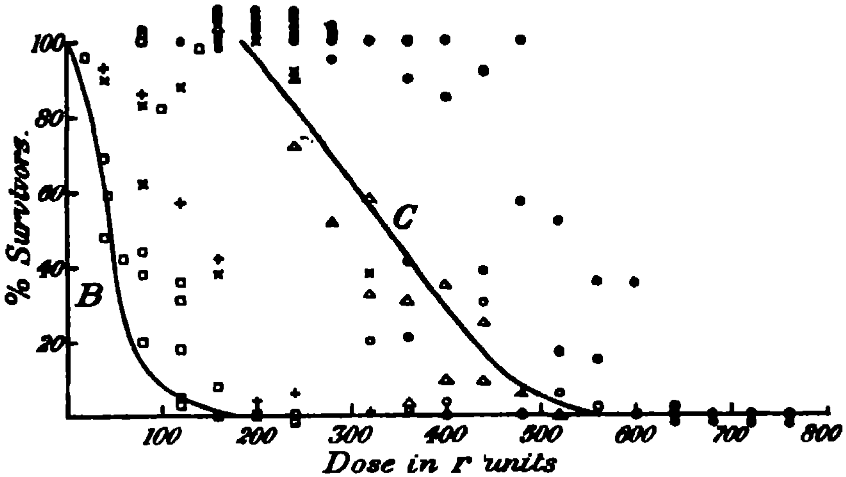

Fig. 5.—Action of X-rays on eggs incubated for times equivalent at 14° C. to: 2·5–3·5 hours, curve B, from fig. 3; 3·5–4·0 hours, □; 4·0–4·5 hours, +; 4·5–5·0 hours, ×; 5·0–6·0 hours, ○; 6·5–7·0 hours, ●; 8·5–9·0 hours, △; 11 hours, curve C, from fig. 3.

These results indicate that between 4 hours and 7 hours of incubation at 14° C. there is a very rapid decrease in the sensitivity of the eggs to irradiation and that after 7 hours the sensitivity increases slightly. The average results as shown in fig. 6 indicate that during the period 3·5–4·5 hours the sensitivity may be reduced to less than a third in as short a time as 30 minutes. The method of egg collection only ensures that the eggs in two different batches do not differ in age by more than 30 minutes. Hence the rapid change in sensitivity fully explains the extensive scatter in the experimental results during this period. Although the results are so scattered yet it is possible to estimate approximately the median lethal dose of irradiation at the various times and these values are shown in fig. 6.

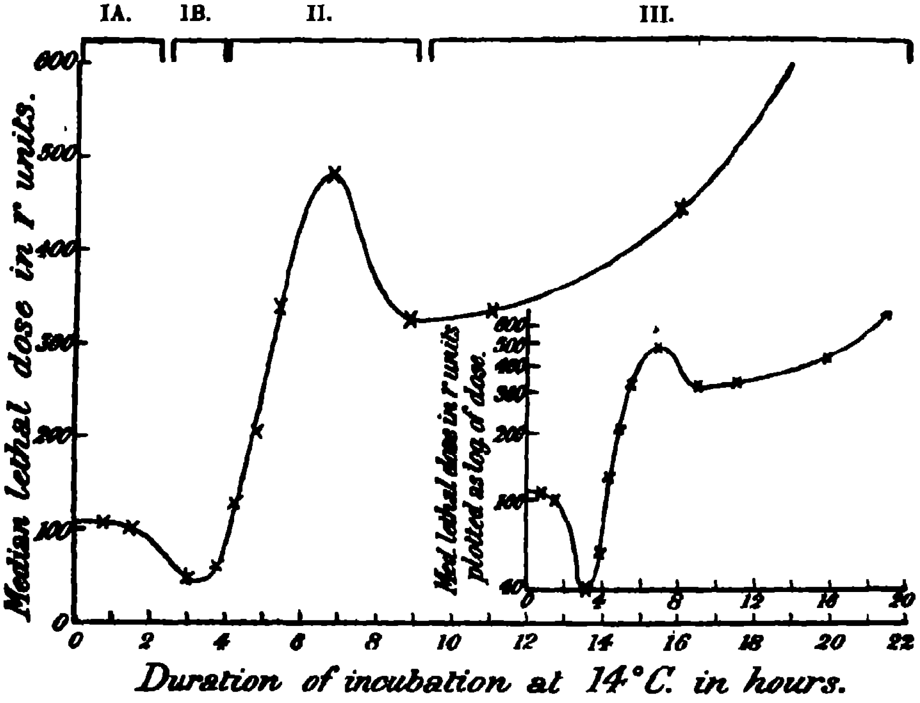

FIG. 6.—Relationship between the median lethal dose of X-rays, the duration of incubation at 14° C. and the stage of morphological development of *Calliphora erythrocephala*. Upper abscissa, I unicellular stage, A central nuclei, B nuclear migration. II blastoderm stage. III stage subsequent to blastoderm invagination.

The Development of the Calliphorine Egg.

The eggs were examined histologically in order to correlate the changes in sensitivity with the grosser features of the development of the embryo. The technique of section cutting is of unusual difficulty and is described in the Appendix.

The development of the egg has been described by Noack (1901) and I have confirmed many of his descriptions and have used his terminology. The calliphorine egg is fertilized as it passes down the oviduct during the actual process of oviposition. The newly laid egg, fig. 7, Plate 6, consists of a mass of protoplasm in which many small yolk granules are suspended. It contains one or sometimes two nuclei which lie in a halo of condensed undifferentiated protoplasm in which there are no yolk granules. The egg is surrounded by a vitelline membrane and an outer shell or chorion. This chorion is very tough and impermeable, but it has an opening at one pole, the micropyle, through which the sperm enters.

The egg nucleus divides, but no cell membranes are formed and the egg remains a multinucleated single cell until the eighth nuclear cleavage, fig. 8, Plate 6, when there are about 500 nuclei. Prior to this stage the nuclei are scattered throughout the cell, but they now become arranged in a layer roughly parallel to the egg surface about half-way between the long axis of the egg and the surface. Shortly afterwards the nuclei migrate to the surface and sections of the egg, fig. 9, Plate 6, show the appearance of trailing streamers of cytoplasm running from the nuclei at the surface towards the interior. The nuclei form a single-tiered layer immediately under the vitelline membrane. Cell membranes begin to form at the periphery and separate the nuclei from each other, but the cells are open to the interior of the egg and their cytoplasm is continuous with a cytoplasmic syncytium shown in fig. 10, Plate 6. At this stage practically all the protoplasm is massed towards the outside of the egg while the yolk granules occupy the inside. Finally the cell membranes are completed to form a single layer of columnar cells, the blastoderm, which surrounds the yolk, fig. 11, Plate 6. A longitudinal invagination of the blastoderm along the ventral aspect of the egg produces a mesodermal tube and shortly afterwards another invagination of the blastoderm occurs on the dorsal aspect near the pole opposite the micropyle, fig. 12, Plate 6. Noack describes this second invagination as endodermal and from it the gut tube develops. Fig. 13, Plate 6, shows a still later stage of development in which the primitive tube of the gut can be clearly seen. The reduction in size of the cells does not allow the other structures to be distinguished.

Rate of Embryological Development.

The relationship between the various stages of development and the duration of incubation is shown in Table II and fig. 6. The egg when incubated at 14° C. remains a unicellular organism for 3·5 hours. During the fifth hour the blastoderm is laid down and the egg becomes multicellular. The first invagination takes place at the ninth hour and the second invagination is complete at the sixteenth.

Table II.—The relationship between the duration of incubation and the degree of morphological development.

Duration of incubation in hours.	Duration of incubation expressed in terms of incubation at 14° C.	No. of eggs examined.	Percentage in each stage of development.						
			Before blastoderm formation, showing—			Transition stage.	After blastoderm formation, showing—		
			No nucleus visible.	1–7 nuclei.	8 or more nuclei.		No invagination.	Invagination	Advanced development.
At 14° C.—									
0·2–0·6	0·2–0·6	76	97	3	—	—	—	—	—
0·8–1·2	0·8–1·2	61	39	58	3	—	—	—	—
1·8–2·2	1·8–2·2	61	20	69	11	—	—	—	—
3·0–3·5	3·0–3·5	79	6	—	—	94	—	—	—
4·0–4·5	4·0–4·5	124	5	—	—	40	55	—	—
5·0–5·5	5·0–5·5	93	8	—	—	—	92	—	—
7·5–8·0	7·5–8·0	68	6	—	—	—	94	—	—
0·5–1·0 at 14° C. followed by 2·2 hours at 23° C.	7·5–8·0	72	8	—	—	—	92	—	—
0·5–1·0 at 14° C. followed by 2·7 hours at 23° C.	9·0–9·5	86	4	—	—	—	8	88	—
9·0–9·5 at 14° C.	9·0–9·5	72	6	—	—	—	—	94	—
1·0–1·5 at 14° C. followed by 3·0 hours at 23° C.	10·5–11·0	100	7	—	—	—	—	93	—
1·0·1–5 at 14° C. followed by 4·5 hours at 23° C.	15·5–16·0	100	5	—	—	—	—	—	95

The rate of nuclear division was calculated in the following way. Sections of eggs which had been fixed during the fifth hour of incubation at 14° C. were examined and in many of them the blastoderm was in actual process of deposition. In 12 longitudinal sections of these eggs the nuclei were counted and the average number was 51. In 12 corresponding cross-sections the average number of nuclei was 12. If it be assumed that the eggs are approximately cylindrical then the number of nuclei in each egg is $51/2 \times 12 = 306$. Eight nuclear divisions are required to produce this number of nuclei and therefore at 14° C. nuclear division occurs about once every half-hour.

Development of the Irradiated Egg.

It has been shown that X-rays can prevent the hatching of the calliphorine egg and the following experiments were devised in order to find whether the irradiation caused an immediate stoppage of development. Eggs from a half-hour laying period were plated and were X-rayed after less than one hour of incubation at 14° C. A dose of 300 r units was given. At this stage of incubation the eggs are unicellular and contain about 8 nuclei. After irradiation the eggs were divided into two groups. One group was incubated till hatching occurred and out of 514 eggs, 1·5% hatched (*cf.* Table I). The second group was incubated at 14° C. for 11 hours and was then fixed and sectioned for microscopic examination. It has been shown, Table II, that unirradiated eggs incubated for 11 hours at 14° C. have reached the stage of infolding of the blastoderm. Sections of 107 irradiated eggs incubated for the same time were examined and 86 or 80% of these had not formed a blastoderm. In 21 or 20% the blastoderm had been laid down, but in none of the eggs was there any sign of invagination. These results show that while at least 20% of the eggs continue to develop after irradiation with 300 r units, only 1·5% actually hatch. It is concluded, therefore, that the effect of irradiation is not to cause the immediate stoppage of development. The estimation of the number of nuclei in eggs before blastoderm formation is difficult, but there was evidence that in many eggs nuclear division had continued after irradiation.

Discussion.

The chief results of this investigation are set forth in fig. 6, which shows the variations in the sensitivity of the developing calliphorine egg to X-rays and also the times of the various stages of its embryological development. During the process of development the sensitivity of the egg decreases greatly, but fig. 6 shows that the process is not continuous but that there is one period (4–7 hours) of very rapid loss of sensitivity and two periods (2–4 hours and 7–10 hours) during which the sensitivity actually increases. The relative extent of the changes in sensitivity are compared in the inset graph of fig. 6 in which the median lethal dose is plotted on a logarithmic scale. The general loss of sensitivity during development is in accord with the Law of Bergonie and Tribondeau, but this law does not explain the detailed course of the changes in sensitivity and in particular cannot account for the two periods during which the sensitivity increases.

The relationship between the sensitivity of the calliphorine egg to X-rays and the degree of embryological development closely agrees with that found in other eggs. Henshaw and Henshaw (1933) described an almost identical curve relating X-ray sensitivity to the development of the egg of *Drosophila*. The developmental changes in the latter egg are almost identical with those in *Calliphora* and the doses of X-rays used by Henshaw and Henshaw were similar to those which I used in *Calliphora*. Glasser and Mautz (1933) obtained comparable results with eggs of *Drosophila*, although they discerned no periods of increased sensitivity. The egg of *Ascaris* also behaves in a fashion similar to that of *Calliphora* for Schinz and Zuppinger (1928) found that the sensitivity of the *Ascaris* egg decreased when the egg changed from the single celled stage to the blastula stage. They found that the sensitivity gradually increased between the blastula stage and the gastrula stage and that thereafter it gradually decreased.

The results of my investigations throw some light on the nature of the action of X-rays on cells. The present conception of the mode of this action depends on three methods of biological measurement. Firstly, the effect of X-rays has been measured by their lethal action, the method used in this investigation. Secondly, the effect has been measured by the genetic mutations which X-rays produce. In experiments of this type, the literature of which has been summarized by Hanson (1933), X-rays not only produce genetic mutations but also produce changes in the shape and behaviour of the chromosomes and the two effects have been correlated. The third method of biological measurement of X-rays is the direct observation of the behaviour of cells which have been irradiated. By this method it has been shown that a cell is most easily killed during the process of nuclear division (Mottram, 1913; Holthusen, 1921; Vintemberger, 1928, *a*) and also that irradiation may prevent nuclear division either temporarily or permanently (Canti and Donaldson, 1926).

The latter two methods of studying X-ray action are mainly concerned with effects produced on the nucleus of the cell and Vintemberger (1928, *b*, and 1929) attemped to show that the nucleus was the primary seat of X-ray damage by the following method. He measured the dose required to kill two groups of developing eggs of the frog. In the one group an attempt was made to screen the cell nuclei and in the other to screen the cytoplasm. He concluded that the nucleus of the cell is enormously more sensitive to the lethal action of X-rays than is the cytoplasm.

The results of my investigation afford no direct evidence of any difference in sensitivity between the nucleus and the cytoplasm, but they afford indirect evidence. Fig. 6 and Table II show that the development of the calliphorine egg involves two phases of cellular activity. During the first phase the segmentation nucleus divides continuously, but there is no cytoplasmic division and the egg is a multinucleate single cell. During the second phase nuclear division occurs, but it is accompanied by division of the cytoplasm and the egg consists of many cells each of which have one nucleus. The change from the uninuclear to the multinuclear phase is abrupt and is accompanied by an enormous and sudden decrease in sensitivity to X-rays, fig. 6. This suggests that the decrease in sensitivity is related in some way to the division of the whole cell rather than to the division of the nucleus alone.

This conclusion is further supported by the fact that there was no significant difference in the sensitivity of the egg during the first 2 hours of development (Table I and fig. 6). During this period nuclear division occurred continuously, but cytoplasmic division did not occur and there was no significant difference between the sensitivity of eggs of less than 1 hour incubation and that of eggs of between 1 and 2 hours incubation.

There is an increase in sensitivity between 2·5 and 3·5 hours and the extent of the increase is shown in the inset graph of fig. 6. At this time the nuclei and the cytoplasm are in process of migrating to the surface of the egg and this process is a preliminary to the change from the unicellular to the multicellular state. The complexity of the change which must occur in the cells at this time is the simplest explanation of the increased vulnerability of the eggs.

This explanation also serves for the increased vulnerability which occurs between 7 and 10 hours. At this period the blastoderm is on the point of invaginating and again the complexity of the cell changes which must precede the invagination process is the simplest explanation of their increased sensitivity to X-rays.

My results and conclusions differ from the results of other workers who have studied the action of X-rays on mitosis. There is, however, an essential difference in the material studied. In the material studied by other workers mitosis was followed by cell division and they measured the action of X-rays in stopping the combined process of nuclear and of cytoplasmic division. Insect eggs are peculiar in that nuclear division occurs for several hours before cell division commences and hence it is possible to compare the action of X-rays on the process of nuclear division and on the process of combined nuclear and cytoplasmic division. Nuclear division begins soon after the egg is laid and continues at a rate which appears to be fairly constant for the first 10 hours. In spite of this fairly constant rate of nuclear activity, the sensitivity of the cells to X-rays varies extensively and erratically. These variations cannot be explained by variations in nuclear activity, but they are correlated with obvious changes in the activity and morphological structure of the complete cell units. These facts cannot be explained by Vintemberger's finding that the action of X-rays is chiefly confined to the nucleus, but they can be explained if the action of X-rays causes greater damage to the complete cell unit when the cell is undergoing a complicated process of differentiation.

The results of this investigation also show that the lethal action of X-rays on the calliphorine egg is not an immediate lethal effect. In spite of their having received a lethal dose of X-rays some eggs continue to develop for a short time before death occurs. A similar effect is produced in *Colpidium* (Crowther, 1926). *Ascaris* (Holthusen, 1927), the embryo chick (Strangeways and Fell, 1928) and on certain tissue cultures (Spear, 1931; Cox, 1931).

The shape of the curves for the action of X-rays in this paper agree closely with those obtained with many other tissues (Packard, 1931; Henshaw and Henshaw, 1933; Crowther, 1926; Canti and Spear, 1927; Cox, 1931). The significance of the shapes of the curves will be discussed later by the author, but explanations have already been put forward by Crowther (1926), Condon and Terrill (1927), Packard (1931), Clark (1933), and the explanation of Packard and Clark, namely, that the characteristic curve of biological action of X-rays expresses the individual variations of the biological units, appears to the author to be the most reasonable explanation of the facts.

I gratefully acknowledge the advice of Professor A. J. Clark, F.R.S., throughout the course of my work. I thank Dr. R. P. Hobson, of the London School of Hygiene and Tropical Medicine, for his detailed advice about the breeding of *Calliphora*; Dr. A. E. Cameron, of the Department of Zoology, Edinburgh University, for his continuous help in the interpretation of the embryology of *Calliphora*; and Dr. W. O. Kermack, of the Laboratory of the Royal College of Physicians of Edinburgh, who instructed me in the methods of statistical analysis which I have used. My thanks are also due to Mr. Kilgour, of the Pathological Department of the Royal Infirmary of Edinburgh, through whose patience and skill I obtained microscopic sections of the eggs of *Calliphora*.

The expenses of the investigation were paid partly by a grant to Professor Clark from the British Empire Cancer Campaign Fund, partly by a grant to me from the Moray Fund of Edinburgh University and from the Medical Research Council.

Summary.

(1) The relationship between the sensitivity to the lethal action of X-rays of the egg of *Calliphora erythrocephala* and the degree of embryological development of the egg has been shown.

(2) The sensitivity of the egg decreases with age, but the loss of sensitivity follows an irregular course.

(3) The variations in sensitivity can be explained by the accompanying changes in the embryological development of the egg.

(4) The evidence suggests that X-rays exert their action on the complete cell unit rather than on the cell nucleus exclusively.

(5) The lethal effect of X-rays on the calliphorine egg is not an immediate effect.

(6) The shape of the characteristic curve of the lethal action of X-rays on calliphorine eggs at various stages of development has been defined.

APPENDIX.

Method of Preparing Microscopic Sections of the Eggs of Calliphora erythrocephala.

By D. KILGOUR.

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No satisfactory method of preparing sections of the eggs of *Calliphora* has yet been described, but the following method succeeds in two-thirds of the attempts. The difficulty of the technique is due to the nature of the chorion of the egg, the hardness and impermeability of which make fixation and section cutting very difficult. The following method gave the best results.

About 200 eggs are immersed in Carnoy-Lebrun fixative for at least 2 hours. They are washed in industrial spirit and after successive dilutions of the spirit in water. The chorion is dissolved by immersing the eggs in sodium hypochlorite solution (about 0·02 gm. chlorine per 100 c.c.) for about 2 minutes. The judgment of the time required to dissolve the chorion without damaging the underlying cells is difficult and requires much practice. The eggs are now thoroughly washed in spirit and then in water. The chorion becomes detached at this time and when this occurs the eggs are washed in ascending concentrations of alcohol. After immersion for 1 hour in absolute alcohol and then for 1 hour in benzole the eggs are embedded in paraffin (melting point 54° C.). Sections are cut and Kull's method of staining gives the best results.

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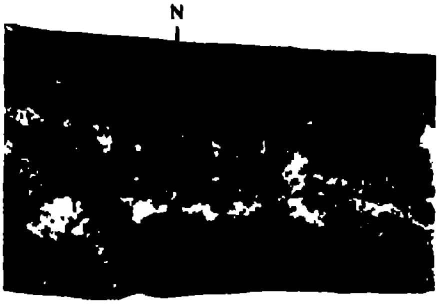
EXPLANATION OF PLATE.

Fig. 7.—A longitudinal section of a newly laid egg of *Calliphora erythrocephala*. × 47. The egg consists of a mass of protoplasm in which fine yolk granules are suspended. No nucleus is visible in the section.



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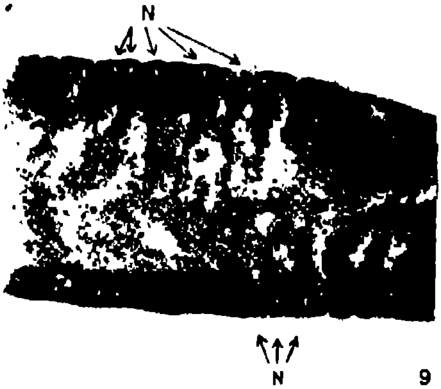

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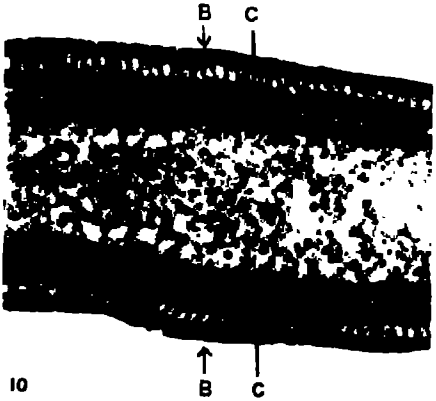

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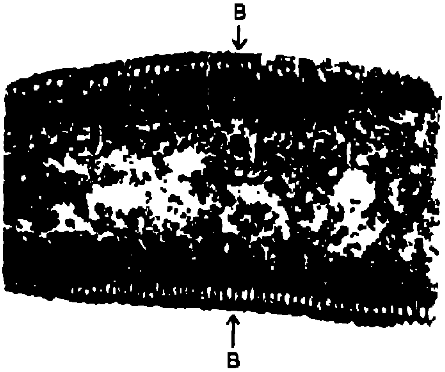

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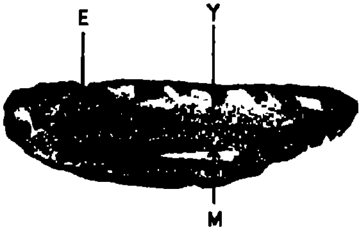

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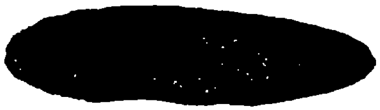

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Fig. 8.—Part of a longitudinal section of an egg incubated for 1·8 2·2 hours at 14° C. × 125. N are nuclei surrounded by cytoplasm which contains no yolk granules. The nuclei are centrally situated.

Fig. 9.—Part of a longitudinal section of an egg incubated for 3–3·5 hours at 14° C. × 125. N are nuclei which have recently migrated to the surface of the egg.

Fig. 10.—Part of a longitudinal section of an egg incubated for 5–5·5 hours at 14° C. × 125. B is the blastoderm. Cell membranes can be seen near the margin of the section. The cells are still open to the interior of the egg and their cytoplasm is continuous with a syncytium of cytoplasm C.

Fig. 11.—Part of a longitudinal section of an egg. × 125, incubated for 7·5–8 hours at 14° C. The blastoderm B consists of a layer of complete, columnar, cells.

Fig. 12.—Longitudinal section of an egg. × 47, incubated for 11 hours at 14° C. The mesodermal tube (M) is shown. The endoderm grows from point E. The yolk (Y) is very much reduced in quantity.

Fig. 13.—Longitudinal section of an egg incubated for 16 hours at 14° C. The yolk (Y) is greatly reduced in amount. The egg consists of a cellular mass in which the gut canal C can be distinguished. × 47.

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The Principle of Formaldehyde, Alcohol, and Acetone Titrations. With a Discussion of the Proof and Implication of the Zwitterion Conception.

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Consideration of the implications of the zwitterion hypothesis of Bjerrum (1923) makes it desirable to state afresh the principles underlying the methods commonly employed in the titration of amino-acids. Deductions of considerable theoretical importance, *cf.*, *e.g.*, Calvery (1933) are still being made on the supposition that the alkalimetric formaldehyde titration method of Sørensen (1907) and the corresponding alcohol method of Foreman (1920) and of Willstätter and Waldschmidt-Leitz (1921) estimate the carboxyl groups of amino-acids whilst the acidimetric acetone titration of Linderstrøm-Lang (1928) estimates the amino-groups. Yet the zwitterion hypothesis indicates that this assumption is the reverse of the truth.

Discussion is greatly facilitated by collective consideration of recent physicochemical evidence clarifying the principles upon which these common biochemical methods rest. In a recent discussion of two of the titrimetric methods (Van Slyke and Kirk, 1933) the existence of this evidence is ignored, so that it becomes necessary to systematize and elaborate the empirical argument of these authors in the light of the relevant investigations of Grünhut (1919), Cray and Westrip (1925), Michaelis and Mizutani (1925), Birch and Harris (1930, *b*), and Levy (1933). At the same time new and useful developments are indicated.

Zwitterionic Constitution of Amino-acids.

The zwitterionic conception was used by Adams (1916) in the discussion of acidity, but was first fully developed by Bjerrum (1923), who pointed out that an amino-acid was comparable in its dissociation behaviour with an ammonium salt except for the fact that the positive and negative ions of the former remained permanently attached to one another by a carbon atom or chain of atoms, forming a zwitterion. This conception, which would remove all doubt as to the allocation of experimentally determined titration constants to the appropriate groups of ampholytes, was supported (*a*) by analogy with related non-ampholytes, *cf.* comparison of aspartic and tartaric acids; (*b*) by the failure of isoelectric amino-acids to exhibit common reactions of the amino-group, such as condensation with formaldehyde; (*c*) by the generally salt-like behaviour of amino-acids.

Collateral evidence in support of Bjerrum's views has come from a number of sources. Thus the effect of change of solvent on the dissociation constants of amino-acids (Michaelis and Mizutani, 1925; Neuberger, 1934), the heats of ionization of amino-acids (or the temperature coefficient of their dissociation constants) (Ebert, 1926; Birch and Harris, 1930, *a*; Greenstein, 1933) and the volume change of ionization of amino-acids (Weber, 1930) all favour a zwitterionic constitution for these compounds.

Bjerrum's own analogy between the physical behaviour of amino-acids and that of salts has been extended by Borsook and MacFadyen (1930) in consideration of the effects of amino-acids on the p_H of phosphate solutions. The effect is composite depending on the effect of amino-acids on (*a*) the ionic strength, and (*b*) the dielectric constant of the aqueous solutions. The former effect is direct evidence of zwitterionic structure, while the latter, too, has recently been shown by Wyman and McMeekin (1933) to be explicable only in terms of the zwitterion. Molecular volumes and water-alcohol solubility ratios (Cohn *et al.*, 1933) have been used to demonstrate the highly charged nature of the amino-acid molecule and considerations of freezing point depression and ultra-violet absorption (Anslow, *et al.*, 1933) are also in accordance with its supposed zwitterionic character. Further discussion of the evidence from salt-like behaviour is given by Cohn (1932).

A less conclusive line of evidence is provided by calculation, from a comparison of $p_{K'_1}$ of glycine itself and $p_{K'}$ of glycine ester, of the induced effects of the groups $-COOC_2H_5$ (assumed identical with $-COOH$) and $-COO^-$ on the dissociation of the amino-group (Ebert, 1926; Miyamoto and Schmidt. 1932). The use of formaldehyde condensation with amino-groups to facilitate a similar study of carboxylic dissociation is rightly rejected by Ebert (1926); the view maintained by Birch and Harris (1930, *b*) that formation of titratable weakly-basic methylene amino-acids actually occurs is incompatible with experimental results, and as pointed out by Levy (1933) the only valid explanation of the effect of formaldehyde is that the latter reacts with the alkaline modification only of the amino-acid, thus depressing the effective concentration and lowering the titration curve.

On the whole therefore it is clear that the chemical and physical behaviour of amino-acids is more readily explicable on the zwitterionic theory than on the older theories of their constitution.

Theory of Acid-base Titration.

Since weak acids and alkalis undergo unit change in p_H for every tenfold change in the ratio of free acid (or base) to neutralized salt, it follows that an acid whose $p_{K'}$ (p_H of 50% neutralization) is 6 will be 9·1% neutralized at p_H 5, or 99% neutralized at p_H 8. This relation makes it clear that accuracy of titration depends on choice of an end-point as far as possible removed from $p_{K'}$, limits to the extension of p_H range of the titration being imposed by the magnitude of the so-called "free acid" (or "free alkali") correction. This is an amount of the solution used for titration in sufficient excess of the requirement for neutralization to produce in the final solution a p_H corresponding to the selected end-point. Its magnitude varies with the concentration of the reagents (Rehberg, 1925), but usually is significant only with end-points acid to p_H 3·5 (or alkaline to 10·5). Yet this limit means that carboxylic groups (aqueous $p_{K'} < 5$) cannot be titrated sharply to an acid end-point since at such an end-point significant amounts of acid will be required to produce even the smallest visible change in indicator colour (see p. 171).

The features of titration important to the present work are (*a*) this systematic impossibility of acid titration of carboxylic groups, especially those of amino-acids, and (*b*) the fact that, whilst aqueous titration implies titration between two p_H values, the formaldehyde, alcohol, and acetone titrations may be carried out using the same indicator for initial and end-points. In order to understand such titrations it is necessary to understand the effect of change of solvent on the dissociation.

Theoretical Effect of Solvent on Acid-base Dissociation.

The strength of an acid in solution, depending as it does on the extent of dissociation, will evidently be favoured by a solvent of high dielectric constant which will diminish the mutual attraction of oppositely charged ions; an actual chemical affinity between solvent and dissociating ions will have a similar effect. Brönsted (1926) states that the dielectric effect is predominant, but the chemical effect cannot be neglected. The idea of the combination of H^+ with water and other hydroxylic solvents in particular, to form oxonium ions (Brönsted, 1928; Sidgwick, 1927) is now being extended to other ions (Wright *et al.*, 1931), and implies that the dissociation of an acid may be regarded as a substitution in which solvent molecules compete with one ion for association with the other. Consider water ($D = 81$), alcohol ($D = 26$) and acetone ($D = 21$); we see that in water mutual attraction will be low and association with solvent high, in alcohol mutual attraction and association with solvent will both be high, in acetone mutual attraction will be high and association with solvent low. The same acid therefore will be strong in water, weak in acetone, and intermediate in strength in alcohol.

In accordance with the general definition of Brönsted (1928) acids are to be regarded as substances capable of liberating H^+ (*e.g.*, HCl, H_3O^+, NH_4^+) whilst bases are substances which can receive H^+ (*e.g.*, Cl^-, H_2O, NH_3); thus all dissociations may be conveniently expressed in terms of the hydrogen ion and directly related to the p_H scale.

Now if we represent the dissociation of an ordinary acid thus:

$$A = H^+ + B^- .$$

that of a divalent acid thus:

$$A = H^+ + B^{--}$$

that of a mono cation (such as NH_4^+) thus:

$$A^+ = H^+ + B,$$

and that of a di cation (such as $NH_3^+ R NH_3^+$) thus:

$$A^{++} = H^+ + B^+$$

we see that the products of dissociation attract one another in the first two cases, have no action in the third and repel one another in the fourth. It follows that lowering of the dielectric constant (as by transfer from aqueous to alcoholic solution) will diminish the strength of ordinary acids, increase that of polycationic acids, and leave that of monocationic acids unaffected.

Mathematically expressed :

$$p_{K_c}(\mathrm{H_2O}) - p_{K_c}(\text{solvent}) = \frac{36{,}000\, z_B}{T}\left(\frac{1}{D_s} - \frac{1}{D_w}\right), \qquad (1)$$

where p_{K_c} is the dissociation constant expressed in terms of concentration, z_B is the valency of the component B, and D_w and D_s are the dielectric constants

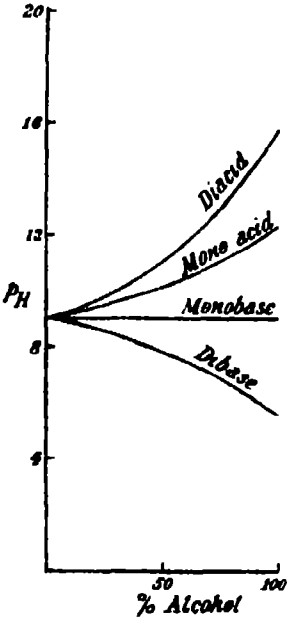

Fig. 1.

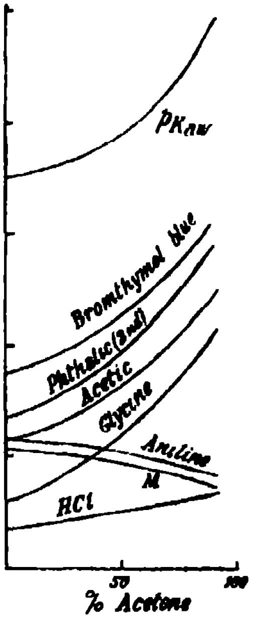

Fig. 2.

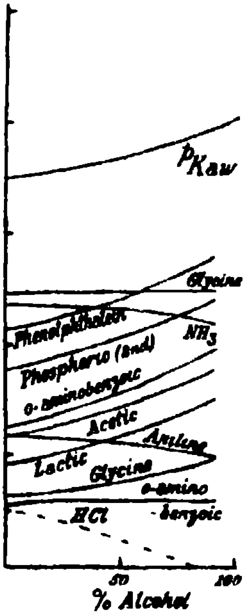

Fig. 3.

Fig. 1.—Theoretical variation of p_{K_c} in alcohol. Fig. 2.—Observed variation of $p_{K'}$ in acetone (Cray and Westrip). M = benzene-azo-α-naphthylamine. Fig. 3.—Observed variation of $p_{K'}$ in alcohol (Mizutani).

of water and solvent at temperature T. By calculation, increase of p_{K_c} for 100% alcohol will be 6·8 units for a divalent acid group, 3·4 for a monovalent acid, 0 for a monobasic ion, and a decrease of 3·4 for a dibasic ion, and so on. Fig. 1 depicts this general behaviour. That this dielectric effect dominates the situation, other effects producing changes of only < 1 p_K unit (Halford, 1931), is exemplified by comparing fig. 1 with the experimental values of figs.

2 and 3. The appropriateness of the equation, however, in examples where B is $> +1$ or < -1, is rendered uncertain by the observation (Neuberger, 1934) that the individual charges of a zwitterion function almost as discrete charges. The same may apply to like charges carried by B, and indeed divalent acids do not undergo so large a change of strength as anticipated. There is, however, some augmentation, since univalent acids are titratable against phenolphthalein in the alkalimetric alcohol technique (p. 152).

Bronsted's use of p_{K_c} emphasizes the diversity of constants in use to interpret acid-base phenomena. p_{K_c} is the classical constant defined by the mass action law, in which the components are expressed as concentrations; it is determined conductometrically, and suffers considerable change with change of ionic strength of the solution, or as above, of the solvent. $p_{K'}$ is the "titration constant," expressed in terms of acid-base concentrations and hydrion activity; it is potentiometrically determined, and varies with ionic strength and solvent in a manner different from the variation of p_{K_c} by the extent of change of the absolute hydrion activity correction. p_K finally is a thermodynamic constant *expressed in terms of activities of the components, so that p_K is* non-variant with ionic strength or solvent.

The thermodynamic relation between $p_{K'}$ values of the same acid in different solvents is, however, incapable of accurate expression owing to the uncertain magnitude of the absolute hydrion activity correction involved, and an arbitrary relation has so far been inevitable (Wynne-Jones, 1933; Neuberger, 1934). It has, however, been pointed out (Halford, 1931), that, since the use of some alternative arbitrary p_H scale involves only the addition of a constant to each $p_{K'}$ value in that solvent, the validity of comparison of these $p_{K'}$ values is not altered by the use of any particular scale. Since our intention is to make such comparisons with a minimum departure from conventional modes of expression, we accept as the arbitrary basis of the p_H scale, the assumption that the p_H of identical concentrations of strong acid (HCl) in two solvents is identical, provided that the solutions are of negligible ionic strength.

This basis, adopted by Cray and Westrip (1925), implies a different arbitrary zero potential for each solvent, the difference being a constant, roughly determinable (*cf.* the relation between potentials measured against the calomel and normal hydrogen electrodes). The procedure has the advantages (*a*) that uncertain junction potentials—up to 4 mv. (Neuberger, 1934)—may be avoided in the comparison of two acids, and especially (*b*) that major change of p_H in an HCl solution on adding alcohol, becomes merely an expression of the effect of dilution. Alternative bases rest upon benzoic acid buffers (Wynne-Jones, 1933), acetic acid buffers (Halford, 1931), and an accepted value for hydrion activity in aqueous solution (Michaelis and Mizutani, 1925; Neuberger, 1934). In these the determined p_H and $p_{K'}$ values are convertible to the basis of our discussion by addition of a suitable constant (*cf.* figs. 2 and 3). For absolute alcohol this has a value approximately + 2·5 (Brönsted, 1928), but is much smaller in aqueous mixtures.

Linderstrøm-Lang (1928), in introducing the acidimetric acetone titration, based his theoretical treatment on the absolute activity coefficient for converting p_{K_c} into p_K values, but as such the treatment cannot be satisfactorily correlated with potentiometric or conductometric measurements in mixed solvents. We have therefore adopted the present formulation for general discussion.

For convenience and to avoid violation of current conceptions, we shall continue to describe the titration of cationic acids (Brönsted) as a titration of "basic groups."

Aqueous Solvents.—In mixed aqueous solvents, ions tend to associate with water rather than the other solvent present, and, so long as there is sufficient water in the mixture, they behave almost as though in aqueous solution. As an instance, Butler (see Hartley, *et al.*, 1932, p. 65) shows that lithium ions behave in certain respects almost identically in water and 30% alcohol. Hartley (p. 24) emphasizes the remarkable effect on dissociation of so little as 0·1% of water in non-hydroxylic solvents. Consequently, determinations of the weakening of strong into weak acids in pure acetone do not imply the same situation in aqueous solvents, and HCl, $RHSO_4$, HNO_3, and the sulphonic acids are still to be considered strong acids in 90% alcohol or acetone.

This behaviour has other importance in that it shows that quantities of up to 20% of formaldehyde in the formaldehyde titration have probably little effect, physically, on the $p_{K'}$ values of the ions in solution.

Ampholytes and Polyvalent Ions.—The simple distinction between dissociating groups carrying different ionic charges provided by alcohol or acetone addition is of great value in allocating measured titration constants to definite groups of an ampholyte, and has been mentioned as evidence of zwitterionic structure. In ions carrying several charges, the charges tend to behave as discrete individuals, a fact which must not be overlooked in drawing conclusions from a pictorial representation of the ionization of ampholytes. The possibility of similar behaviour in polyvalent anions and cations requires extended systematic investigation. In the meantime, Brönsted's treatment admirably defines the more obvious effects to be expected from alcohol or acetone addition.

Change of Temperature.—The effect of temperature on $p_{K'}$ values is not directly analogous to that of alcohol addition. Although the dielectric constant of water is changed by temperature (from 88 to 78 between 0 and 25°), the product DT of equation (1) remains almost constant.

Observed Change of Acid Strength in Aqueous Acetone.

Pring (1923) showed electrometrically that monovalent bases (aniline and derivatives) underwent no large change of $p_{K'}$ in 90% acetone. Cray and Westrip (1925), from the same laboratory, subsequently determined changes of indicator and buffer strengths in this solvent. Numerous sulphonphthalein and azo indicators, as well as hydrochloric, phthalic, acetic, and chloracetic acids, and glycine, were examined. Selected results are shown in fig. 2. Measurement was made with quinhydrone electrodes, in an electrode chain using 90% acetone throughout (see MacFarlane (1931) for possible oxidation changes), and the general conclusions are these. Monovalent carboxylic acids are weakened 4 to 5 units in 90% acetone (D = 26) and the second group of phthalic acid by 6 units, while the monovalent basic ions undergo a shift of 1 to 1·5 units in the opposite direction. HCl is a strong acid in both solvents, having its p_H changed only to the extent expected from dilution. The ionic product, K_{aw} (*i.e.*, $H^+ \times OH^-$), for acetone-water differs from that of water ($p_{K_w} = 14$), the values found by Pring (1923) being $p_{K_{aw}} = 15 \cdot 5$ at 20° in 50% acetone, and $p_{K_{aw}} = 19 \cdot 7$ in 90% acetone. The so-called "neutral-point" of 90% acetone is therefore at p_H 9·7. The greater rate of change of $p_{K_{aw}}$ in the more concentrated acetone solutions is not unexpected.

Observed Change of Acid Strength in Aqueous Alcohols.

With alcohols, a more extensive survey has been made by Michaelis and Mizutani (1924, 1925) and Mizutani (1925). $p_{K'}$ values for every 10 or 20% change of alcohol concentration were recorded for numerous monocarboxylic and dicarboxylic acids, for phenols, phosphoric and carbonic acids, for ammonia, aniline, and their methylated derivatives, glycine, aminobenzoic acids, and several one-colour indicators. Potential measurement was made with a hydrogen electrode (alcoholic)—reference electrode (aqueous) chain, so that for reasons already discussed, the resulting measure of "p_H" in the alcohol solutions has not its conventional relation to hydrion concentration, even apart from the uncertain junction potentials involved. Selected results are depicted in fig. 3. Change of $p_{K'}$ by this procedure was, as expected, much less than the corresponding effect in the acetone experiments. Monovalent acids, including phenols, were weakened by 2 to 2·5 units in 90% ethyl alcohol (D = 30), the divalent acids by slightly more although studies were not extended beyond the 80% alcohol. The $p_{K'}$ values of monovalent basic ions suffered a decrease of about 1 unit. Investigation of amino-acids has recently been made by Neuberger (1934). No data have been found for $p_{K_{aw}}$ of 90% alcohol, but MacFarlane and Hartley (1932) give 18·7 for 100% alcohol so that the apparent value for 90% alcohol may well be about 16 (18·7 − 2·5).

In methyl alcohol (90%, D = 38), there was a tendency towards decreased $p_{K'}$ values as compared with ethyl alcohol (Mizutani, 1925), the acids being weakened by only about 2 units. For 90% propyl alcohol no data have been found, but the pure alcohol (D = 22) was found by Hunt and Briscoe (1929) to depress the dissociation of weak acids much further than did pure ethyl alcohol.

Observed Change of Basic Function in Aqueous Formaldehyde.

The action of formaldehyde has been studied electrometrically by Birch and Harris (1930, *b*), using a similar method to that of Michaelis and Mizutani for alcohol. They made observations in varied concentrations of formaldehyde up to 16% on ammonia, propylamine, aniline, phenol, acetic and boric acids, and numerous typical ampholytes. Ammonia, propylamine, and aniline, had their p_K's lowered by 2% formaldehyde addition ; phenol, acetic and boric acids were not significantly affected by 5% formaldehyde. Ampholytes, selected with reference to their zwitterionic behaviour, showed, according to the number of amino or acidic groups present, groups depressed or unaltered by formaldehyde addition. Levy (1933) has formulated more precisely the change in the second $p_{K'}$s of α-amino acids. Selected results are reproduced in fig. 4. Change of $p_{K'}$ varied, for concentrations above 1%, as log $(CH_2O)^2$ for α-amino acids, or as log (CH_2O) for proline. This implies that each α-amino group was associating, not with one formaldehyde molecule as suggested by Sørensen, but with two, and that proline associated with one. Depression of $p_{K'}$ in 10% formaldehyde was about 2·5 to 3 units, but with tyrosine and glycine large specific variations were obtained.

Formaldehyde, Alcohol, and Acetone Titrations.

These titrations depend simply on selecting certain groups for titration which are altered in $p_{K'}$ by addition of one of these reagents, and using an indicator which is not so altered, or *vice versa*. The principle may be applied at sight to the estimation of acid or basic groups of any given strength, where it is not limited by lack of appropriate indicators (see Clark, 1928).

It is impossible to enumerate the conceivable variations, but figs. 5 and 6 depict, in condensed form, a few examples showing the variation of $p_{K'}$ of acids, bases, and indicators with solvent concentration, upon which titration depends. In fig. 7 these effects are given in detailed form for glycine in 90% acetone or alcohol and 10% formaldehyde. As this diagrammatic presentation in the customary form is self-explanatory, it remains just to point out that figs. 5 and 6 simply represent the change of the mid-points of the curves depicted in fig. 7. Then, let A in fig. 5 (or fig. 7) depict an acid group, B a basic indicator; if A is approached from the alkaline side by titration in water with HCl B will be at the mid-point of its colour change before A is 1% titrated; if 90% acetone is added, A will now be almost completely titrated before B reaches the mid-point of its colour change. This is the principle of the Linderstrøm-Lang titration (1928) using benzene-azo-α-napthylamine as

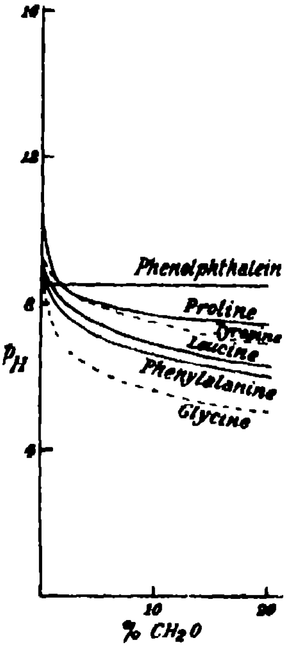

Fig. 4.

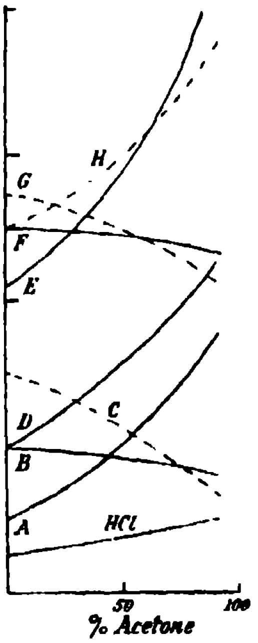

Fig. 5.

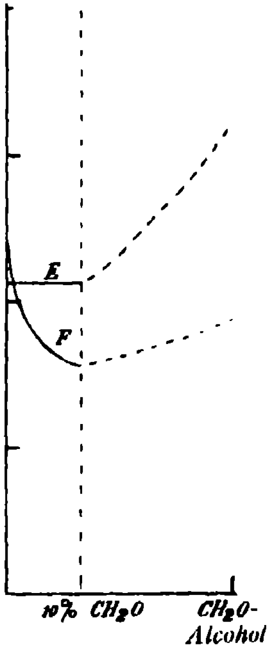

Fig. 6.

Fig. 4.—Observed variation of $p_{K'}$ in CH_2O (Levy). Fig. 5.—Basis of acetone (or alcohol) titration. Fig. 6.—Basis of formaldehyde titration.

indicator (see fig. 7); improved accuracy would follow the use of a dibasic indicator C of more alkaline range, if such were known.

Again, let F (figs. 5 or 7) be a basic group, E a one-colour, divalent (or multivalent) acid indicator; if F is approached from the acid side by NaOH titration in water, E is coloured before F is significantly titrated; after alcohol addition F must be largely titrated before E can become coloured. This is the principle of the Foreman (1920), Willstätter (and Waldschmidt-Leitz, 1921) alcohol titration with phenol- or thymolphthalein. But, if H is a monovalent acid not significantly titrated in water, it will also be considerably titrated in 90% alcohol before the indicator changes colour, and will introduce error into the method (see p. 152). A monovalent indicator would improve the specificity by not titrating such acid groups, but would decrease the quantitativeness; suitable indicators are, however, rare. Lastly, if G is a divalent basic group of larger p_K. than F, it will nevertheless be satisfactorily titrated. This explains the satisfactory estimation of the second lysine group by alcohol titration (Willstätter and Waldschmidt-Leitz, 1921).

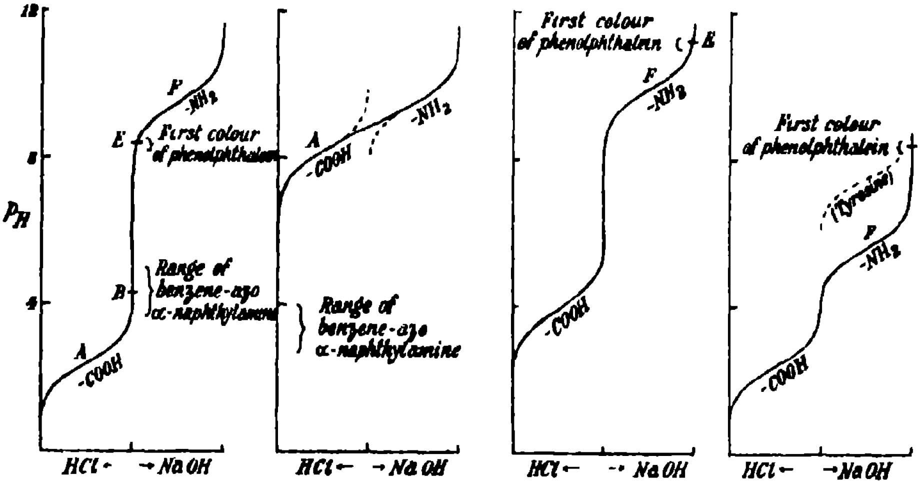

FIG. 7.—Effect of solvent on glycine titration curves.

(*a*) in water; (*b*) in 90% acetone (Linderstrøm-Lang titration); (*c*) in 90% alcohol (Foreman titration); (*d*) in 10% formaldehyde (Sørensen titration).

Alternatively, let B (or C) of fig. 5 be a basic ion mixed with an acid group D, A an indicator of the same valency as D; if B is approached from the acid side, A will be at its end-point in water when B and D are only 1% titrated (it will, however, be a vague end-point owing to the presence of "free acid");

if 90% acetone is added, B will now be almost completely titrated before A is at its mid-point, but D will still be only 1% titrated. Titration, specific for the basic group, is thereby accomplished. Again, it may be wished to estimate HCl in the presence of the acid group A. In water, this is impossible since A is already 50% estimated by the time residual HCl is reduced to 0·01 N; in 90% acetone, however, the HCl can be largely titrated before A is affected. This is a valuable method for estimating "free HCl" (p. 170).

Fig. 6 (or 7) depicts a simple example of the formaldehyde titration (Sørensen, 1907) and formaldehyde-alcohol titration (Foreman, 1920). F is an amino group, E an acid indicator whose range is presumably not altered by formaldehyde addition; in water E becomes coloured before F is significantly titrated by NaOH; on adding formaldehyde, however, F is now largely titrated before E is coloured; by adding alcohol, this effect is accentuated owing to the changed range of the indicator, but at the same time the concentration of formaldehyde is diminished, reducing the depression of F. There is no electrometric data available to show the resultant effect precisely.

It is apparent in the above that quantitative and specific titration depends not only on the agent used, but on the group to be titrated, and the appropriate selection of the indicator. In previous sections, full demonstration of some of the relevant points has been given, or inferred, as follows: (*a*) titration accuracy varies with the p_H range available for titration, and is a maximum if the titrated group is midway between the titration end-points (see p. 162 for further discussion); (*b*) extension of the available p_H range in water is limited by the magnitude of the "free acid" or "free alkali" corrections, which become significant at about p_H 3·5, or p_H 10·5 respectively; (*c*) in alcohol or acetone an extended p_H range is available for titration, the alkaline limits occurring at p_H 14 or 16·5 respectively; (*d*) the p_H range effective for titrating an individual group, as a result of the relative displacement of groups, is about 5 units for acetone, 3·5 units for ethyl (methyl) alcohol, and 2·5 to 3 units for formaldehyde. This corresponds to a quantitative accuracy of 99·3%, 96%, and 90% respectively, under optimum conditions of titration; (*e*) the zwitterion hypothesis is emphatically confirmed by available critical methods.

The *Linderstrøm-Lang acetone titration* (1928) is then a measure of carboxylic groups (as curve A, fig. 5). It is not specific for amino acids, but it can be used to estimate all other such moderately strong acid groups as happen to be present—oxalates, phosphates, pyruvates, citrates (1st group), tartrates and similar groups, and creatine—while the weaker malate, citrate, lactate, and acetate groups, etc., can be estimated to the extent that they are untitrated by

HCl at the initial point in water. The method therefore should never be applied to complex biological fluids,* but if used on more simple solutions can be expected to be quantitative as it offers a wide p_H range for titration. The alkalimetric *alcohol and formaldehyde titrations* provide a measure of amino groups, but can never be considered quantitative as they do not offer sufficient p_H range for titration; as a consequence, their accuracy mainly depends on an appropriate choice of indicator. They have been discussed in detail as one-indicator and two-indicator methods elsewhere (p. 164). The alcohol titration is not specific for amino groups, but partially titrates magnesium and weak monovalent acids as well. For simple solutions, quantitative accuracy would be increased by the use of acetone in place of alcohol.

Insolubility.—The great practical disadvantage of acetone and alcohol titrimetric methods, that of titrating in solutions which may be quite turbid as a result of precipitation, has been discussed by Waldschmidt-Leitz and Künstner (1927) and by Sørensen and Walther (1928). Waldschmidt-Leitz and Schaffer (1926) who also considered the problem use methyl alcohol as added solvent where phosphate buffers are present.

New Applications.

With the principles thus outlined, other applications may be made. Thus, sodium acetate for use in buffer solutions may be sharply titrated in 90% acetone with standard HCl, using neutral red as indicator. Or again, the strong inorganic acid in a mixture of HCl with phosphoric acid, or in a peptic digest, or in gastric contents, can be titrated by NaOH with satisfactory accuracy, using naphthylamine orange in 90% acetone (p. 170). Again, the titration of any basic group in the presence of acids of similar $p_{K'}$ or *vice versa* may be accomplished by appropriate choice of indicator.

Other valuable applications appear in the allocation of titration constants to the titratable groups of ampholytes. The method was used with advantage (Richardson, 1933) to distribute correctly the three observed titration constants of 2-thiolhistidine, between the four possible titratable groups. It may also be used with nicotinic acid ($p_{K'}$ *ca.* 3·3, 4·8) to show that these groups are respectively the cyclic nitrogen and carboxylic groups, since only the latter is titratable with a suitable basic indicator and acetone.

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* Van Slyke and Kirk (1933) have just examined its application to blood. They observe the effect of lactic acid, etc., but do not consider interference by phosphoric acid, creatine, or phosphoric acid complexes.

Discussion.

It remains to apply the present procedure to the interpretation of a few typical problems. The first of these concerns the titration of the dicarboxylic and the basic amino acids. If aspartic acid and lysine are titrated to a phenolphthalein end-point in formaldehyde, alcohol, or acetone, the former amino acid requires two equivalents of NaOH, the latter only one. These titres are in the ratio of the carboxylic groups of these amino acids, and were regarded as evidence that carboxylic groups were being titrated, contrary to the zwitterionic view. Correspondingly, if HCl titration is made to a benzene-azo-α-naphthylamine end-point in acetone, one and two equivalents respectively are required, giving apparent indication of the titration of amino groups. These results, however, arise from the use of isoelectric, rather than neutralized, amino acids, the titrations being quite ambiguous as evidence. Had the titrations commenced at p_H 7 to 8, lysine would have required two equivalents, aspartic acid one with NaOH, as was demonstrated by Grünhut (1919) using litmus; one and two equivalents, respectively, would have been required with HCl. These titrations are now in the proportions required by the zwitterion hypothesis.

The situation with aspartic acid and the three basic amino-acids is represented in the p_H titration curves of fig. 8 (which is slightly formalized by merging all the acid titration curves, and the two alkaline curves of histidine and aspartic acid). Arginine, lysine, histidine, and aspartic acid are isoelectric at points A, B, C, and E, respectively; titration by NaOH in alcohol, etc., will therefore neutralize respectively 0 (the guanidine group is too strong for titration), 1, 1, and 2 equivalents; titration after neutralization to p_H 7 to 8 (points D and C, *i.e.*, titration of arginine and lysine hydrochlorides, histidine, and sodium aspartate) will require 1, 2, 1, and 1 equivalents. Back titration to methyl red in alcohol (Harris, 1923) is merely equivalent to titration after neutralization to p_H 7–8. Neutralization to litmus results in the estimation of 1·3 equivalents of histidine (Grünhut, 1919); for amino groups incompletely estimated under standard conditions (see p. 164). On the other hand, titration by HCl in acetone will require 2, 2, 2, and 1 equivalents, but after neutralization 1, 1, 2, and 2 equivalents. If the preliminary material were arginine dihydrochloride, histidine hydrochloride, or aspartic acid hydrochloride, etc., corresponding correction has to be made. This interpretation of fig. 8 from the zwitterionic standpoint accords completely with all experimental results as distinct from theoretical conclusions of Sørensen (1907), Grünhut (1919), Foreman (1920), Willstätter and Waldschmidt-Leitz (1921), Harris (1923), and Linderstrøm-Lang (1928).

Protein Hydrolysis Experiments.—Finally, the legitimacy of those chemical studies of enzyme activity purporting to demonstrate equivalent liberation of —COOH and —NH_2 groups during proteolysis must be re-examined. These studies, summarized by Sørensen and Walther (1928), continued by Calvery (1933), apply titrimetric or Van Slyke analytical methods to proteins undergoing enzymic hydrolysis, to estimate the extent and nature of proteolytic scission at given time intervals. The question at once arises whether by comparing amino-N (Van Slyke) with amino-N (alcohol or formaldehyde), and not with —COOH as these authors conceived, results of value are achieved. The answer is that by a fortunate coincidence due solely to the conventional method of titration and not generally applicable, the comparison of these estimations remains valid evidence as to whether acid groups are, or are not, produced in excess of amino groups. Although the argument is a little involved, it may be well to examine in what way analysis of protein hydrolysates differs from others, and how protein workers arrived at accurate conclusions when their theoretical interpretation was inaccurately conceived.

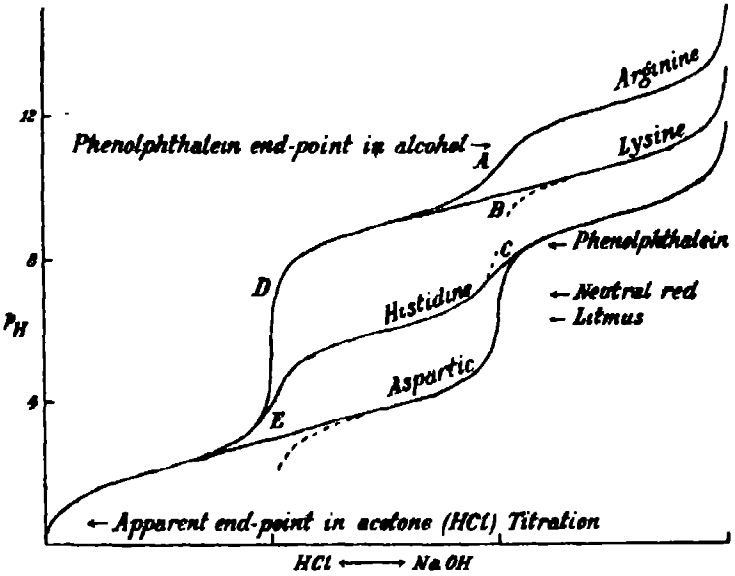

FIG. 8.—Titration curves of trivalent amino-acids.

The crux of the argument lies in the following self-evident statements. (1) Since results of proteolytic analysis are expressed as difference values, only such groups as appear during proteolysis are included in the difference estimations. These appear primarily from some type of peptide scission, but may conceivably result also from ester or other scissions. They are the only groups that must be considered in this discussion. (2) By peptide scission, moderately strong acid and basic groups appear simultaneously in solution, and naturally appear mutually ionized as the ions of a salt, which is a substituted ammonium acetate and has corresponding properties

$$R.CO.NH.R' = R.COO^- + NH_3R'^+.$$

(Frequently two zwitterions actually appear from an original zwitterion, but the only *new* groups in the solution, the only effects which concern the mass estimation, are those having the effect of a salt.) By ester scission, a moderately strong acid group appears free in solution,

$$R.COOR' = R.COOH + HOR'.$$

(3) At the end-point of alcohol and formaldehyde (NaOH) titration, ions of basic groups are converted to the free bases, those of acid groups remain as ions,

$$CH_3COO^- + NH_4^+ + NaOH = CH_3COO^- + Na^+ + NH_4OH.$$

At the end-point of acetone (HCl) titration, ions of acid groups are converted to the free acids, those of basic groups remain as ions,

$$CH_3COO^- + NH_4^+ + HCl = CH_3COOH + NH_4^+ + Cl^-.$$

(4) The titration methods may be applied in two ways: (*a*) by preliminary neutralization before addition of alcohol, etc., and (*b*) by adding alcohol, etc., directly to the un-neutralized digest. The second method is the more usual.

If method (*a*) is used, then, in whatever way newly hydrolysed groups appear in solution, they are present at the initial points neutralized as salts, and are converted to free acids (by HCl) or bases (by NaOH) at the end-points. Consequently, primary and secondary amino-N is estimated by alkalimetric alcohol and formaldehyde titration, and moderately strong acid groups by acidimetric acetone titration—in contrast to the original interpretation of these titrations. Thus, where alcohol and formaldehyde titrations are compared with amino-N (Van Slyke or colourimetric) this provides merely a comparison of the efficiency of the methods. The data of Harding and MacLean (1916) on tryptic protein digests thereby serve only to show that amino-N (formaldehyde) is 5 to 30% low compared with concordant values for amino-N (Van Slyke) and amino-N (ninhydrin), and does not give the believed comparison of —COOH and —NH_2 proportions. Titration methods in this form are coming into general use to estimate amino-N, and the question of their relative analytical worth is discussed elsewhere (p. 162).

If, on the other hand, method (*b*) is used, then hydrolysed groups are titrated from the form in which they are liberated in solution, to the form in which they are found at the end-point. Thus the splitting of a normal peptide bond to liberate equivalent amounts of positive and negative *ions* as salts, will be estimated equally by acidimetric acetone titration, or alkalimetric alcohol or formaldehyde titration. But the scission of ester bonds to liberate *free acid groups* will not be estimated by acidimetric titration, of which the end-point is the free acid ; it will, on the other hand, be estimated by alkalimetric titration, of which the end-point requires the anion of the acid group. Thus by method (*b*), formaldehyde or alcohol titration will include total amino-N of peptide bonds (= total acids of peptide bonds) + total acid groups appearing free (this will be true irrespective of incidental reaction of the free acid groups with the buffers of the digest, since this merely leaves other weaker acids to be neutralized). Evidently, in this peculiar case of unneutralized protein digests, groups equivalent to total acid groups appearing by scission will be titratable. Conversely, acidimetric acetone titration, in these particular circumstances only, will be of such dimensions as to represent total amino-N. It was described in these terms by Linderstrøm-Lang (1928), who fails, however, to emphasize that it is a specific case without general validity.

Another distinction between methods (*a*) and (*b*) is that titration of the salts liberated from peptide (or acyl proline) scission by the former method is a one indicator method, whereas titration by the latter method represents a two indicator titration from the p_H of the neutral salt to a chosen end-point. As such it is increasingly quantitative the more alkaline the end-point, and is thus in contrast to titration by method (*a*) (see p. 164). It is the growing tendency to apply conclusions from the protein method (*b*) to biological analysis by method (*a*) that has necessitated our re-statement of the principles involved.

To summarize, the titration of protein hydrolysates varies in interpretation according to whether the appearing groups are neutralised first to the chosen indicator or not. In the former method (*a*), alkalimetric alcohol and formaldehyde titration estimates amino-N, not acid groups as has been believed, and acidimetric acetone titration estimates acid groups, not amino-N. In the latter method (*b*), free acid groups become wedged, as it were, into the alcohol and formaldehyde titres making them of dimensions equivalent to total acid groups, as has hitherto been accepted. The similarity of the argument with that applicable to trivalent amino-acids (fig. 8) may help to elucidate it.

Applications.—Proceeding to general discussion of the estimation of particular scissions by various methods, ordinary scission of a peptide bond will be measured equally by estimating —COOH groups (acetone) or amino-N (Van Slyke, colourimetric, alcohol, or formaldehyde) with minor discrepancies due to particular analytical defects of the respective methods. Scission of an acyl proline bond will be fully measurable as —COOH (acetone), in large measure as amino-N (alcohol and formaldehyde), but not at all as amino-N (Van Slyke). Scission of ester linkages will be measurable by method (*b*) by formaldehyde or alcohol titration, but not by acetone titration or Van Slyke analysis; scissions liberating free amino groups would be estimated by the latter methods and not the former. $R_2PO_4^-$ will be indistinguishable as an acid group from $RCOO^-$, and scission of its esters (if this does occur, Levene and Hill, 1933) will appear to be carboxylic ester scission.

Evidence of Sørensen and Walther (1928) that increase of alcohol and formaldehyde titre is virtually the same as that of acetone titre during 80-hour hydrolysis of casein, gliadin, and gelatin, is therefore valid evidence that ester (and similar) linkages have not been hydrolysed to any extent comparable with hydrolysis of peptide (or acyl proline) bonds. By an electrometric method, this has been confirmed for gelatin with an accuracy of about ± 1 linkage per molecule (Cannan and Muntwyler, 1930).

Likewise, evidence of Waldschmidt-Leitz and Künstner (1927) showing virtual equivalence of Van Slyke and alkalimetric alcohol estimations during 3 days peptic hydrolysis of histone, ovalbumin, casein, and gelatin (in spite of 9 and 23% of (proline + oxyproline) in the last two proteins) is presumptive evidence that only peptide bonds are split by pepsin. Bergmann, Zervas, and Schleich (1932) continued a tryptic hydrolysis of gelatin with erepsin (amino-polypeptidase), and showed that disparity between Van Slyke and alcohol estimations attributable to acyl-proline scissions then appeared. It is therefore difficult to interpret obervations of Waldschmidt-Leitz and Künstner (1927), and of Calvery (1933) that *complete* enzymic hydrolysis of histone and of ovalbumin (as controlled by acid hydrolysis) was accompanied by equivalence to within 3%, with no disparity appearing during ereptic hydrolysis, when ovalbumin, at least, contains over 4% of proline (neglecting oxyproline). Although numerous explanations are possible none is very acceptable, and good reason is provided for preferring titration methods whose mode of action is predictable to the Van Slyke method in such experiments.

It is therefore desirable to predict the effect of a few hypothetical scissions on the titrimetric methods. Of these, the liberation of free basic groups, or of phosphoric acids, has been considered, but other types of scission, not entirely speculative, are suggested by the demonstration that arginine of proteins only becomes accessible to decomposition by arginase (Hunter, 1925) or by H_2O_2 (Edlbacher, 1924) after proteolysis. If a linkage, R . NH . C(: NH) . NH . CO.R′ be postulated, its hydrolysis to form the salt of the strong base will not be estimated by alkalimetric alcohol or formaldehyde titration, since the strong base remains in salt combination at the end-point, whereas by acidimetric acetone titration the acid group will be estimable as usual. Converse effects would be observed if strong (sulphonic) acid groups should happen to be hydrolysed from a type of peptide combination. From ester combination, these acids would increase the alkalimetric titrations by method (*b*) as usual, but will actually decrease acidimetric acetone titration (method (*b*)) since their hydrolysis is equivalent to adding strong acid. Weak basic (histidine, chromoproteins) or weak acid groups, if liberated as salts (*i.e.*, where basis $p_{K'}$ is larger than accompanying acid $p_{K'}$) will be indistinguishable by method (*b*) from amino or carboxylic groups. If liberated free, or as mixed acids and bases too weak to form salts, they will usually not be estimated at all.

Recommended Protein Titration Methods.—Although previous discussion has aimed so to explain the principle of the titrations that particular applications can be devised to cover particular cases when they arise, one obvious recommendation might be missed. *All that is necessary to demonstrate equivalence of acid and basic groups liberated during proteolysis of soluble proteins is a simple aqueous titration at given time intervals to* p_H 6 *to* 6·5, the p_H of minimum buffering power of protein-polypeptide-amino acid mixtures. Scission of ordinary acid-base linkages, having the effect of introducing a salt of which the above p_H is the neutral point, will not influence such a titration. Only acids and bases appearing free in the solution will be determined. By titrating thereafter by the Linderstrøm-Lang (acids) or Willstätter (amino-N) methods, or determining amino-N (Van Slyke), disparities representing scission of proline or arginine linkages, etc., or factors yielding approximate polypeptide content, may be determined. To minimize indicator masking when aliquots are not available for titration, bromcresol purple (5·2–6·8) will serve for aqueous titration if Linderstrøm-Lang titration is to follow, or propyl red (4·6–6·6) if Willstätter titration follows. Titration will be facilitated by the well-known Lüers-Grünhut comparator technique.

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Summary.

The principles upon which the formaldehyde, alcohol, and acetone titrations of amino acids depend have been discussed in the light of the zwitterion hypothesis and the evidence for this hypothesis summarized.

The Sørensen and the Foreman (Willstätter) titrations estimate amino groups, the Linderstrøm-Lang titration carboxylic groups, contrary to accepted convention. In the particular case of protein investigations, however, the old comparison of amino-N (Van Slyke) with Willstätter titrations to demonstrate equivalent liberation of acid and amino groups by enzymic hydrolysis remains valid, by a fortunate coincidence resulting from the modified titration procedure conventional in these studies.

Useful new applications of the general principles have been suggested.

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Critique on the Biological Estimation of Amino Nitrogen.

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For the estimation of amino nitrogen in complex fluid mixtures three typical methods are available. These are (*a*) the nitrous acid method (Van Slyke, 1911, 1912), (*b*) the formaldehyde titration (Sørenson, 1907; Malfatti, 1909; Lüers, 1920), and (*c*) the alcohol titration (Foreman, 1920; Willstätter and Waldschmidt-Leitz, 1921). Yet by general agreement none is entirely satisfactory.

Method (*a*) is tedious in multiple determinations, is subject to fortuitous error from frothing, and from reducing agents present in solution. It is known to give faulty values for glycine, cystine, tryptophane, arginine, lysine, and glutamine (Schmidt, 1929; Plimmer, 1924; Chibnall and Westall, 1932), and for various peptides (see Thierfelder and von Cramm, 1919; Hopkins, 1929; Dunn, Butler, and Deakers, 1932). As discussed later, it gives a partial estimation of ammonia, urea and allantoin, creatinine, and the aminopurines. It is not influenced by proline (Van Slyke, 1911). Methods (*b*) and (*c*) have much in common. They are both rapid in use, but are of limited accuracy owing to a difficult end-point in complex solution, and of restricted scope since they must be confined to almost colourless extracts. Their quantitative defects are to be discussed, but the methods are best applied serially only to solutions of closely similar chemical composition. As a final option, colourimetry (Harding and MacLean, 1916; Riffart, 1922; Folin and Wu, 1922) is both too uncertain and too difficult to apply to chemically complex, coloured solutions.

Methods (*b*) and (*c*) were developed and applied originally, largely with the very specific purpose of studying enzyme and protein chemistry. It was natural, therefore, that the very considerable success they there achieved should have encouraged their adoption in estimating amino-N analytically, and the formaldehyde titration in particular has long been applied to this end. Yet analogous extension of alcohol (or acetone) titration to this end is, from first principles, liable to serious error. The consequent likelihood of frequent deceptive conclusions based on incautious application of these methods (and already in evidence, Foreman (1928), Rahn (1932)) suggested the need of further study. Moreover, though alcohol and formaldehyde (NaOH) titrations are described in protein studies as estimating carboxylic groups, this is completely incorrect as a general statement, since these titrations, applied analytically, measure amino groups (p. 129). Likewise, the Linderstrøm-Lang acetone titration (1928), described as measuring amino groups actually measures acid groups. It has therefore been falsely applied to blood analysis by Zirm and Benedict (1931). Obviously, considerable confusion exists as to the principles involved.

It has seemed timely, therefore, to examine critically a series of data obtained upon single biological extracts by each of the methods (*a*), (*b*), and (*c*), and to examine the ultimate bases of the methods. Conclusions have thereby been reached in such a way as to outline the proper scope of each method and the considerations which restrict its general use.

Experimental.

Preparation of Material.—A suitable raw material of considerable biochemical interest has been sought in the aqueous extracts of plant leaves, for obviously, no conclusion as to the primary synthesis of proteins and protein material can be attained without a degree of certainty as to the amino-N content of such material.

For general use a stock extract was prepared from young cocksfoot, which was rapidly dried, finely chopped, and thrice extracted by bringing to 95° with 10 volumes of water in a water bath. After filtration through muslin, the extract was evaporated *in vacuo* to a suitable bulk, one volume of alcohol stirred in, and the mixture centrifuged from precipitated protein and inorganic material (see "alfalfa filtrate," Vickery, 1924). There was thus obtained a permanent stock solution containing relatively simple molecules. 3 kg. of dried grass yielded 8·4 litres containing 2·4 gm. total soluble N per litre (total soluble N = 0·67% weight of dried grass). For use 750 c.c. alcoholic stock were concentrated to small bulk to remove the alcohol, and made to a litre with water; successive preparations showed no significant alteration of amino-N (Van Slyke) content.

For runner bean, broad bean, lucerne, and brussels sprouts extracts, fresh leaves were minced, and the cell sap pressed out in a Buchner press. Then protein material was denatured at 95°, filtered off on muslin, and the solution concentrated and stored under toluene. All extracts had to be decolourised by the use of selected methods before titrimetric analysis.

Methods of Analysis.—Data recorded are the mean of comparable duplicate analyses. Total N was determined by the micro-Kjeldahl steam-distillation technique, amino-N (Van Slyke) by the Van Slyke micro-apparatus (10 minutes reaction at 15–18°), ammonia-N by vacuum distillation from magnesia at 40° (Chibnall and Westall, 1932). Amide-N was determined as increase of ammonia-N after 2 hours hydrolysis in boiling N H_2SO_4, peptide-N as increase of amino-N (Van Slyke) after 16 hours hydrolysis in boiling 5 N H_2SO_4. Nitrate-N was determined as increase of ammonia-N after reduction with acidified reduced iron (Vickery and Pucher, 1929). Ammonia present in the extract was, in general, not removed before analysis for amino-N. p_H was determined at a Hildebrand hydrogen electrode freshly plated for each determination.

Titratable-N was estimated under a variety of conditions, but all estimation was on a micro scale comparable to the Van Slyke micro estimation. Titration followed arbitrarily standardized procedures suited to routine analysis in an ordinary laboratory. Therefore stock solutions consisted merely of ordinary 40% formaldehyde, 95% alcohol, and CO_2-free NaOH from protected microburettes. To minimize CO_2 contamination, titrations were rapid and small conical flasks were used to reduce air space. Indicator (phenolphthalein where not otherwise mentioned) was added as a 0·5% alcoholic solution, one drop per 5 c.c. To 2 c.c. of the relatively colourless solution for estimation, N NaOH was added to approximate the end-point, and the solution then titrated with 0·1 N NaOH to the first visible colour as compared with a blank of similar alkalinity. One volume of 40% formaldehyde or 10 to 12 volumes of alcohol were next added, and the solutions immediately titrated with N/35 aqueous NaOH or 0·05 N 90% alcoholic NaOH, respectively, to a similarly defined end-point. End-points were often vague and of a masked colour, care always being necessary to reproduce in the colour blank any change of colour in the natural indicators of the extract. Nevertheless, estimations were generally possible to ± 0·02 c.c. Titres were finally corrected for the acidity of the added formaldehyde or alcohol (as estimated by a blank on 2 c.c. water) and are taken to represent titratable amino groups.

Results.

As preliminary to the investigation, Table I gives the distribution of nitrogen in the aqueous cocksfoot extract, as prepared by removal of alcohol and dilution, and in an extract of young, fresh broad-bean leaves. It is apparent at once that alcohol titration has certainly included in considerable amount some unknown substance not estimated in the Van Slyke apparatus. To study this discrepancy further, however, it was necessary to possess more quantitative data as to the effect of decolourizing the extracts, since Table I presents titratable-N on decolourized material, other data on original material. Where analyses are performed throughout on decolourized material, the disparity is even larger.

Table I.—Nitrogen distribution in the aqueous extracts. % total soluble N.

Extract.	Total N (gm./litre).	Ammonia -N %	Amide -N %	Nitrate -N %	Amino-N.		Peptide -N %	Rest -N %
					Van Slyke %	Alcohol titration %		
Cocksfoot	1·84	5·8	7·2	3·3	31	42*	8·1	44·5
Broad bean	1·02	2·0	18·6	2·9	38	44*	---	38·5

* After lead precipitation.

Methods of Decolourizing the Extract.

Decolouration by Dialysis.—It is generally considered that colour in leaf extracts is associated with material of high molecular weight. Separation of these larger molecules from smaller ones of more interest to analysis was attempted using the three compartment electrodialysis method of Foster and Schmidt (1923). It was hoped that separation of the unknown titratable material might likewise be effected. Unfortunately, effective migration necessitates dialysis from rather acid or rather alkaline solution in order to convert the neutral zwitterions to suitably charged ions, so that systematic errors may accrue from unsuspected hydrolysis, or again from oxidation or reduction induced at either electrode. Nevertheless, some investigation of the trend of migration was desirable.

The general technique of Foster and Schmidt was adopted with little change —wide carbon electrodes, cooling coil maintaining 30° to 35°, CO_2 stream, etc. A current of 1 to 1·2 amps., 210 volts was used. The centre compartment received 150 c.c. of the aqueous cocksfoot extract, the side compartments 100 c.c. of distilled water. During any run, the p_H of the extract was maintained with 8 N H_2SO_4 at a definite red to thymol blue paper (*ca.* p_H 1·3), or with 8 N NaOH at a strong blue to thymolphthalein (*ca.* p_H 10·5). Protein membranes were not permanent under these conditions, cellophane or parchment paper membranes being necessary. Incidentally, there was in the anode compartment a definite odour of hypochlorites during electrodialysis, even though the extract contained only its natural chlorides; in the cathode compartment, steady dilution was proceeding. At intervals during dialysis, the experiment was interrupted, the volume of dialysate measured, and an aliquot removed for analysis.

It was found that electrodialysis at p_{H} 10·5, by which migration of amino acids to the anode might have separated them not only from the colourstuff but also from the inorganic bases, was quite impracticable. In a three-hour cellophane dialysate, the anodic concentration of transported amino-N never exceeded quite a low value, although the colourstuff was already migrating in amounts seriously masking the titration end-point. Meanwhile, all trace of some 15% of the original amino-N (Van Slyke) had been lost. Clearly, anodic destruction of amino groups, as implied by the presence of hypochlorites, was proceeding as they migrated.

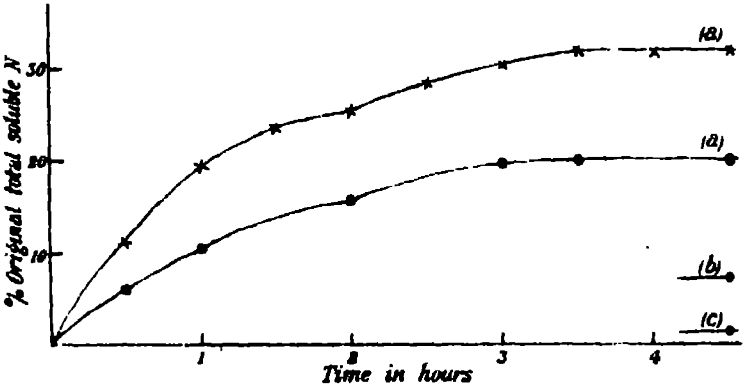

FIG. 1.—Rate of transport of amino-N (Van Slyke and alcohol) p_{H} about 1·3. × alcohol titration; ● Van Slyke. (a) cathode; (b) centre; (c) anode.

Acid electrodialysis (p_H 1·3) to separate amino groups and other bases from the colourstuff gave better, yet still insufficient separation. In fig. 1, a typical experiment through parchment paper is depicted, the ordinates representing total transport of amino-N. At the two-hour interval, the outer dialysates were replaced by distilled water, as their titration end-points were already considerably masked by migration of the colourstuff. This migration of the colourstuff as an ampholyte removes any possibility of its separation from amino acids by electrodialysis, except perhaps by membranes of graded porosity.

Acid stability of the extract was measured by controls at room temperature in both 0·1 N and N free HCl. In 4 days, the ammonia had increased by 15%

and 20% respectively, in 18 days by 25% and 45%. Amino-N (Van Slyke) underwent no significant change. Acid hydrolysis during an experiment will therefore not cause any significant error. In Table II, the distribution of N in the ultimate dialysates (4·5 hours), corrected for removal of aliquots, is recorded. Electrodialysis from acid solution has thus resulted in the transference to the cathode of the bulk of the ammonia-N and the amino-N, but of only about one-half of the total N or amide-N. The residue is presumably of large molecular size, or is un-ionized at the p_H of dialysis.

Table II.—Nitrogen distribution after electrodialysis of cocksfoot extract. % original total soluble N. p_H about 1·3.

	Total N.	Ammonia-N.	Amide-N.	Amino-N.	
				Van Slyke.	Alcohol titration.
Anode compartment	6	0·5	0·5	1·3	2·2
Centre compartment ...	32	0·4	2·7	7·2	7·7*
Cathode compartment..	48	4·4	2·4	20	32
Total	86	5·3	5·6	28·5	42
Original	100	5·8	7·2	31	42*

* After lead precipitation.

Because of the slow and incomplete transference of amino-N, the partial co-transference of the colourstuff, and the possibility of acid hydrolysis in certain extracts, electrodialysis is not recommended in routine analyses unless more extensive investigation can be made. The material measured by alcohol titration but not by Van Slyke analysis is transferred by electrodialysis as a base.

Decolouration by Adsorption.—Attempted adsorptions of the colourstuff upon kaolin, kieselguhr, doucil, silica gel, aluminium silicate gel, powdered and freshly precipitated alumina gave unpromising results. Satisfactory decolouration was obtained with norite charcoal, and details appear in Table III.

25 c.c. of aqueous cocksfoot extract were mixed with suitable amount of HCl or NaOH and the p_H determined. 1 gm. of norite was stirred in, centrifuged, two subsequent 1 gm. norite additions made and the whole centrifuged. The charcoal residues were twice washed and the washings mixed with the supernatant fluid, which was then analysed. Satisfactory decolouration did not occur in the neutral or the rather gelatinous alkaline solutions. At

p_H 2·6 there was partial decolouration, and at p_H 1·1 admirable decolouration, even with 2 gm. norite in hot solution. No particular care was observed in reproducing the technique, but the results show a general loss by charcoal addition of 40% of total N, and 25% of amino-N, irrespective of the extent of decolouration. Since the extent of adsorptive loss is thus high, whereas decolouration is restricted to strongly acid solution—always a latent cause of hydrolysis—no other experiments using closer controls were attempted. It is interesting to recall that Bang's belief (1915) that smaller losses are obtained by charcoal adsorption from 20% alcoholic solution is due rather to the beneficial effect of alcohol on his formaldehyde titration—of which it was an early demonstration—than to any intrinsic effect on adsorptive loss. It is found finally that the source of the discrepancy between alcohol and Van Slyke estimations has not been removed by adsorption.

Table III.—Decolouration by adsorption on norite. N of treated extracts given as % original total soluble N.

p_H.	gm. norite.	Colour.	Total N.	Amino-N.	
				Van Slyke.	Alcohol titration.
			(100)	(31)	
1·1	1	Brown	89	27	—
	2	Pale brown	74	25	41
	3	Pale yellow	58	23	38
1·1 (warm)	2	,,	—	25	43
2·6	3	Pale brown	62	24	37
4·4	3	Brown	57	23	—
5·8	3	,,	65	26	—
8	3	,,	63	22	—
10	3	,,	59	22	—

Decolouration by Bleaching.—Excess of $Na_2S_2O_4$ produced only partial decolouration. NaOCl reacted as would be expected (Langheld, 1909) with considerable effervescence and decolouration, and progressive rapid loss of amino-N. H_2O_2 also reacts destructively with amino-N (Breinl and Baudisch, 1907).

Decolouration by Precipitation.—Addition of $BaCl_2$ or baryta in aqueous or 50% alcoholic solution gave ineffective decolouration. Decolouration by $AgNO_3$, combining the effects of precipitation, and of adsorption on AgCl, was also unsatisfactory. Decolouration by lead acetate was effective within a controlled p_H range.

Sørensen and Hansen (1907) suggested precipitating AgCl from acid solution *in situ* as an admirable method of decolouration without N loss. We have applied it to the cocksfoot extract at p_H 0·6, 1·1, 2·6, 4·7, and 8 using the specified concentrations of $BaCl_2$ (0·16 N) and successive additions of $AgNO_3$ (up to 0·13 N). Acidification itself caused some precipitation and decolouration, which was extended by $BaCl_2$ addition and again by each successive $AgNO_3$ addition. Optimum conditions were at p_H 2·6; decolouration at p_H 4·7 and 8 was sensitive to smaller additions of $AgNO_3$, but was accompanied by bulkier precipitates and greater loss of amino-N. Yet no decolouration of the brown solution further than to a clear yellow was possible, and this was accomplished with loss of at least 10% of amino-N (Van Slyke). There thus appeared to be no special advantage attaching to this method.

The use of lead acetate and basic lead acetate as colour precipitants has long been an established technique, but any control of p_H appears to have been merely automatic depending on the use of the neutral or the basic salt. Britton and Meek (1932) have shown that interaction between lead acetate and NaOH occurs within the p_H limits 6 to 8, and that precipitation is not incipient until nearly two equivalents have been added. From their data, basic lead acetate (0·01 N) may be seen to have a p_H of 7·4, and lead acetate a p_H of 5·9, while at any other p_H the ratio of neutral salt, basic salt, and hydroxide may be determined. Therefore mixed lead acetate salts, appropriately neutralized by this procedure, were added to grass extracts of known p_H, and the effectiveness of decolouration at any p_H observed. As general procedure, 4 c.c. of saturated (2 N) lead acetate solution were added to 25 c.c. of the grass extract with correct neutralization, made up to 40 c.c., and allowed to stand about 30 minutes. The flocculum was centrifuged off, the supernatant liquid acidified acid to p_H 4 and treated with H_2S. The black PbS precipitate was centrifuged off, the supernatant liquid measured for volume and analysed. Where the H_2S treatment produced a browning of the solution, this colloidal suspension was removable by adding a little lead acetate and re-precipitating. As precipitates were not washed at this stage, calculation was made on the assumption that the total solution volume after decolouration was equivalent to the sum of the added volumes. The small systematic error thus introduced (5 to 10%) was corrected away by standardizing the mean data for p_H 5·5 to 6·0 against the data of Table V obtained with washed precipitates.

Results are tabulated in Table IV. The p_H values quoted "5·3 to 5·8" are calculated values resulting from the direct addition of lead acetate (p_H 5·9) in varying concentration to grass extract (p_H 4·8) without p_H adjustment, and are included just to show the effect of lead concentration. Increasing lead concentration produced progressive loss of colour and of total N, but maximum loss of amino-N was reached even in the diluter lead solutions. This was true of both lead acetate and of basic lead acetate (p_H 7·4). Maximum decolouration in 0·2 N lead solutions occurred (with constant nitrogen loss in this case) in the p_H region 5·8 to 7·4. It may be fortuitous that this is the p_H region of lead acetate—basic lead acetate mixtures, but acid regions where basic lead acetate does not exist or alkaline regions where lead acetate does not exist (and the extracts are too gelatinous for reproducible precipitation) were certainly not so effective for decolouration. Precipitation at p_H 5·8 is preferred, since dicarboxylic amino acids are precipitated at p_H 7·4 but not at 5·8. The presence of up to 20% of alcohol until after precipitation (experiments marked *) did not influence the general conclusions. In confirmation of Table I, the unknown titratable material is not removable by lead acetate precipitation.

Table IV.—Decolouration by lead acetate precipitation. N of treated extracts given as % original total soluble N.

p_H.	Reagent.	Lead conc. (N).	Dry wt. Pb ppt. (gm.).	Final vol. (c.c.).	Final colour.	Total N.	Amino-N.	
							Van Slyke.	Alcohol titration.
						(100)	(31)	
2·0	HNO_3	0·2	1·66	30	Yellow-brown	--	31	51
3·3	$HClO_4$	0·2	—	38	,,	79	33	49
3·5	HAc	0·2	1·70	32	,,	88	31	48
4·0*	$HClO_4$	0·2	--	33	,,	76	28	47
5·3 to 5·8	--	0·06	0·60	—	Brown	86	29	46
		0·12	0·80	—	,,	82	26	43
		0·19	0·98	—	Yellow-brown	80	26	44
		0·3	1·34	--	Yellow	76	26	43
5·5*	–	0·2	0·94	34	,,	69	26	42
5·8	NaOH	0·2	0·95	31	V. pale yellow	71	27	44
5·8*	,,	0·2	—	33	,,	71	24	41
6·0	,,	0·1	0·85	30	,,	74	26	43
		0·2	0·87	28	,,	75	26	42
6·5	,,	0·2	1·01	27	,,	—	---	39
7·0	,,	0·2	1·19	27	,,	73	26	36
7·4	,,	0·07	0·27	--	Brown	86	27	40
		0·1	0·61	--	,,	82	27	40
		0·13	0·92	—	Yellow	77	27	36
		0·2	--	31	Pale yellow	72	26	39
		0·2	—	30	,,	75	27	36
9·5	,,	0·2	0·13	31	Yellow-brown	85	25	35
9·8	,,	0·2	--	29	,,	76	27	42

Conclusions.—Of many methods tried, satisfactory decolouration of cocksfoot extract for titration was obtained only by norite adsorption and lead acetate precipitation. p_H optima for these decolourations occurred at p_H 1 in the former, p_H 6 in the latter. Loss of total N or amino-N (Van Slyke) accompanying these decolourations was 40 and 25%, or 27 and 17% respectively. The lead acetate method also has the advantage of removing phenolic and phosphoric groups capable of interfering in the titrations (see later). The AgCl precipitation method gave no complete decolouration, and a loss of 10% amino-N (Van Slyke). By electrodialysis methods, amino groups migrating to the anode compartment underwent destruction.

The Alcohol Titratable Material.

It has become clear that there is present in the extract in considerable quantity some material which is estimated by alcohol titration but not by Van Slyke analysis. It is found that the substance is not adsorbed or precipitated by the usual clarifying agents or by 50% alcohol, and migrates under acid electrolysis as a base. It therefore becomes of first importance to ascertain whether the result is due to a nitrogenous base, and if so, to amino-N not estimated by Van Slyke analysis.

Fractional Precipitation.—The technique of the fractionation of aqueous plant extracts into nitrogeneous sub-groups has been described by Vickery (1924), and has been followed in principle here. Detailed description is not given as results did not have the relation to the investigation that was anticipated. The procedure was to treat litre quantities of the stock aqueous extract progressively with lead acetate, Neuberg's reagent, and phosphotungstic acid, and analyse the resulting fractions (after removal of the reagents) for total N, amino-N (Van Syke), and amino-N (alcohol). Only those fractions which contained the titratable unknown (the supernatant liquid —" filtrate "— in all cases) were further fractionated. All precipitates were removed on the centrifuge and washed at least three times. Concentration of the solutions after fractionation and removal of the reagents was carried out at 40° *in vacuo* until suitable nitrogen concentrations for microanalysis were obtained. Total N on all filtrates subsequent to Neuberg fractionation was determined by Pucher, Leavenworth, and Vickery's method (1930) to include nitrate-N. Lead acetate used for decolouration was added in the proportions defined above, at a p_H yellow-orange to methyl red (*ca.* 5·8). Titration of phosphotungstic acid filtrates after attempted removal of the acid with baryta or amyl alcohol-ether was impossible owing to a masking blue alkaline colour.

It was readily accomplished, however, after a further lead acetate precipitation.

Analytical data for two of the fractionations are presented in Table V. The figures give only the general trend of nitrogen distribution, as reproducibility would require considerable experience of the lengthy precipitations involved. In particular, it seems that original material has been consistently lost on the precipitates discarded during the manipulation (Table V includes the careful analytical data of Vickery (1924) for alfalfa as a partial check on these losses). Yet in spite of this it is at least clear that the titratable unknown is neither an amino group precipitable by the Neuberg reagent, nor a nitrogenous base precipitable by phosphotungstic acid. Of the material, some has been lost in the lead acetate precipitate (the bulk of whose nitrogen is of quite unknown chemical type (Vickery and Vinson, 1925) and was not traced owing to the intense masking colour. Yet in the main, the material simply escaped precipitation entirely.

Table V.—Distribution of nitrogen after group fractionation. N given as % original total soluble N. Italics = data of Vickery for alfalfa.

	Total N.			Amino-N (Van Slyke).			Amino-N (alcohol titration).	
Original N	100	—	—	31	—	*36*	*ca.* 54	—
N of lead precipitate .	12	—	*11*	3·4	—	*5·4*	*ca.* 12	—
,, lead filtrate	72	—	*85*	26	—	*29*	42	—
,, Neuberg precipitate .	52	51	*58*	26	29	*25*	28	28
,, Neuberg filtrate	15	11	*24*	1	1	*3·1*	8	9
,, PT acid precipitate .	2	5	—	0	0·1	—	0	0
,, PT acid filtrate .	—	7	—	—	0·4	—	—	8
,, final lead filtrate ...	—	8	—	—	0·5	—	—	6

Weak Acids, Ureides, etc.—Although the alcohol titration technique is considered to be sensitive only to basic groups, partial estimation of weak acids such as phenols and certain ureides is not excluded (p. 129). It can be expected where any monovalent acid is being titrated against a multivalent acid indicator (*e.g.*, phenolphthalein) of similar p_H range. In Table VI, the magnitude of the partial titration—using phenolphthalein (p.p.) p_H 8·5, or thymolphthalein (t.p.), p_H 9·5—of suitable acids in 0·05 M solution, is indicated. $p_{K'}$ values are quoted from the inadequate literature, and are approximate only. Chlorogenic acid was from coffee beans, hydantoin and uracil were kindly supplied by Professor C. R. Harington and Dr. H. R. Ing, other samples were commercial products. The increase of titre by alcohol addition is most clearly demonstrated when the acid is monovalent and when the indicator change-point occurs on the acid side of the group titrated.

The water soluble phenols most commonly discovered in plants (Wehmer, 1931; Link and Walker, 1933) are gallic, salicylic, chlorogenic, and protocatechuic acids, catechol, and quinol. Considered from our viewpoint, gallic acid is destroyed in alkaline solution too rapidly for titration, salicylic acid is too weak ($p_{K'}$ 13) and chlorogenic acid somewhat too strong (Table VI) to be titratable. Of acid ureides found to occur, allantoin, xanthine, theobromine, and theophylline may be presumed titratable, but only the first was sufficiently soluble in water for examination. Hydantoin and uracil were other soluble acid ureides. These ureides, titrating definitely in alcohol, gave no positive formol titration. HCN, finally, is another weak acid occasionally found in plants, whose behaviour has been examined by Ringer and Grutterink (1926).

Table VI.—Estimation of weak acids by alcohol titration. Titres given as % one equivalent. Procedure exactly as for amino-N.

	Molarity.	$p_{K'}$ (weak).	NaOH titre (aqueous).		Increase NaOH titre (in 85% alcohol).	
			To p.p.	To t.p.	To p.p.	To t.p.
Carbonic acid	0·05	10·2	100	112	0	0
Catechol	0·05	9·5	9	50	50	20
Uracil	0·05	9·5	10	50	70	40
Hydantoin	0·05	9·1	13	65	60	30
Protocatechuic acid	0·05	9·0	123	175	15	5
Allantoin	0·025	8·9	40	80	45	13
Chlorogenic acid	0·05	8·5	150	197	25	2

The distribution of such substances during the fractionations above should now be considered. They would remain in the centre compartment under acid electrodialysis, unless coupled as an ampholyte with some basic group when they would migrate to the cathode. In the group fractionations, the phenols, except quinol, were precipitable by lead acetate; but the ureides examined were not so precipitable. The phenols, except quinol, were precipitated by mercuric acetate with or without addition of sodium carbonate as for Neuberg precipitation (quinol was oxidized under Neuberg's conditions); the ureides gave a cloudiness, soluble in excess, but were precipitated on addition of carbonate; in a quantitative test on allantoin almost complete precipitation was obtained. Phosphotungstic acid has been found an effective precipitant for purines, pyrimidines, and glyoxalines where these have been tested (Drummond, 1918; Peters, 1930). Consequently it is clear that neither phenols nor ureides could have been present in the final filtrate of the group fractionation to induce the excessive alcohol titre found there. On the other hand, phenols might well be a factor in the excessive titre of the material precipitated by lead acetate. Electrodialysis has thrown no light on the matter because of the necessary lead precipitation of the undialysed residue.

Finally, by carrying out a phosphotungstic acid precipitation directly upon the aqueous cocksfoot extract, and examining the fractions after lead decolouration, it was again confirmed that excessive alcohol titre was not due primarily to basic or ureide material, nor to phenols.

Acid Hydrolysis.—Hydrolysis in 5 N H_2SO_4 for 16 hours, followed by removal of NH_3 with NaOH and of Na_2SO_4 with alcohol, concentration and decolouration by lead acetate, gave no indication of the cause of the excessive alcohol titre. Nitrogen figures corresponding to 30% of the original total N as amino-N (Van Slyke), and 46% as amino-N (alcohol) were obtained after hydrolysis, as compared with 26% and 42% for decolourized extracts (Table V) without hydrolysis.

Titration of Ashed Extracts.—By process of elimination it was now increasingly clear that the bulk of the excess alcohol titration must be due to inorganic material. Data of Britton (1932) on the titration of salts with alkalies pointed either to magnesium salts or to alkaline aluminates as a source of the trouble, the alkaline earths being too basic to interfere. In direct alcohol titrations of these salts, no sharp end-points could be obtained since the precipitated solid phase hindered rapid attainment of equilibrium. Yet it was soon shown that alkaline earth salts did not interfere, that alumina titrated to no more than 5% after addition of alcohol, and that *magnesium salts were titrated* to about 75% (p.p.) or 85% (t.p.) by the adopted alcohol titration technique. It was therefore to be expected that the so-called "amino-N" determined by alcohol titration would persist in part in an ashed extract.

Aliquots of the stock cocksfoot extract were ashed, the ash dissolved in approximately N HCl (or HNO_3), and titrated. Alcohol titres of ash "amino-N" corresponding to 10·5% (and 11%) of the original total N were obtained. This compared with an average figure of 15% for the excess alcohol titre (Tables IV and V) so that most of the excess was certainly due to inorganic material. Indeed, if the magnesium of the dissolved ash were precipitated as magnesium ammonium phosphate, an estimate of 0·31 gm. magnesium per litre extract was obtained, corresponding in terms of "amino-N" to exactly 14·5% of the total N. Total ash amounted to 7·9 gm. per litre, or

430% of the total soluble N. In another example—an aqueous extract of perennial rye-grass containing ash as high as 1500% of the total soluble N—"amino-N" titres on the ash equivalent to 30% of original total N were obtained. Ashed extracts gave no formaldehyde titration.

Soluble Magnesium.—Since there is now strong evidence that the excess alcohol titre is due in very large measure to soluble magnesium, it is necessary to demonstrate that any chosen extract contains sufficient magnesium to account for the excess titre. Several different leaf extracts were selected, clarified with lead acetate or norite, and concentrated to a given volume. Aliquots were taken for nitrogen analysis. The remainder was ashed, dissolved in acid, and analysed for magnesium by precipitation of magnesium ammonium phosphate. Estimated magnesium was assumed to be equivalent to $\frac{14}{12\cdot16} \times \frac{75}{100}$ (= 0·86) parts by weight of "amino-N." It should be noted that the estimation of magnesium by alcohol titration is the ordinary behaviour to be expected of it as a base, and correlates perfectly with the zwitterionic interpretation of the alcohol titration of amino acids as a titration of basic groups. The titration is incomplete because magnesium is too strong a base for satisfactory titration by the procedure standardized for these experiments. Although precipitation occurs during titration, titration is not intimately dependent upon it. This viewpoint is much more consistent than that of Willstätter and Waldschmidt-Leitz (1923) who, finding that their method, believed to titrate acid groups, is able also to titrate magnesium, consider this to be due to complete insolubility of $Mg(OH)_2$ in the solvent.

Table VII.—Correlation of magnesium content with excess alcohol titre. Data given as weight % of amino-N (Van Slyke) of treated extracts.

Extract.	Magnesium.	"N" equivalent of Mg.	Alcohol titration "N."
Cocksfoot extract, treated with $PbAc_2$	65	56	159
Cocksfoot extract, treated with norite	73	63	156
Vickery's "alfalfa filtrate," treated with $PbAc_2$	80	69	163
Lucerne III, treated with $PbAc_2$	56	48	141
Broad bean II (dried leaf), treated with $PbAc_2$	49	42	154

Table VII depicts the results. The alfalfa filtrate was an original sample kindly supplied by Dr. H. Vickery. In only one extract of the five was there distinctly insufficient magnesium to account for the observed increase of alcohol titre above the ideal 100%. This extract was a broad-bean extract, notable for the appearance of masking colours during titration, rendering the end-points uncertain. Nevertheless, more extracts should be examined to establish surely that sufficient magnesium always occurs in an extract to account for the excess alcohol-titratable "amino-N."

Comparative Titrations.

Numerous minor modifications of the two titration principles quoted in the introduction are to be found. Careful consideration is required before these may be applied indiscriminately. Most frequently, these modifications have been based on evidence afforded by titrating pure solutions of single amino acids from their isoelectric point in water to some different chosen end-point after formaldehyde or alcohol addition. This can afford a true control only when working conditions are such that the titration is made from an indicator approximating to the isoelectric range of amino acids in water to some different chosen end-point after formaldehyde or alcohol addition.

Such methods have been used with the formaldehyde titration by Henriques and Sørensen (1910) using litmus paper and phenolphthalein (p.p.), and by Lüers (1920), Grünhut (1919), and Northrop (1926), using neutral red (n.r.) and p.p.; Tillmans and Kiesgen (1927) have used n.r. and thymolphthalein (t.p.) for the alcohol titration. The methods necessitate, as confirmed by the authors, complete removal of phosphates, carbonates, and any other groups titrating partially between p_H 7 and 9, which would otherwise be included in the titration. Lüers, especially, by his carefully applied formaldehyde method, obtains data for cereal extracts in which the interference can be clearly seen, as *aqueous* titres between n.r. and p.p. in the untreated extract are often larger than the *total formaldehyde* titre (between n.r. and p.p.) in the phosphate-free extract. Likewise, the two-indicator titrations of Harris (1923) from t.p. in alcohol back to m.r. (methyl red) still in alcohol is liable to interference, not only from phosphates, etc., but even from overlapping of the weaker carboxylic acids.

To avoid interference by phosphates, etc., many workers prefer to use only one indicator, titrating to it first in water, and then again after addition of formaldehyde or alcohol. Where this is done, no claim as to quantitative accuracy based on the titration of single amino acids as above can be made, as it has no reference to working conditions. This inherent defect should not be forgotten if applying analytically such methods as the formaldehyde methods of Malfatti (1909) and Cole (1928), or the alcohol methods of Foreman (1920) and

Grassmann and Heyde (1929). Nevertheless these titres will bear an approximately constant ratio to true amino-N, whereas two-indicator methods will vary fortuitously according to the presence of phosphates, etc. A more detailed discussion of these effects is given later. In the meantime, the great diversity of conditions which have been used is illustrated in Table VIII by a résumé of some of the methods used in biology.

Table VIII.—Résumé of Methods.

Author.	c.c. extract.	Reagent (concentration before titration).	Indicator (concentration before titration).	Initial p_H.	Final p_H (apparent).	Alkali.
One-indicator titrations—						
Malfatti (1909)	10	CH_2O till maximum titre *ca.* 8%	p.p.	First red	First red	N/14 NaOH
Foreman (1920)	5 (10)	85% alcohol	0·0011% p.p.	First red	First red	0·1 N alc. KOH
	5 (10)	70% alcohol in 2·5% CH_2O	0·0009% p.p.	First red	First red	0·1 N alc. KOH
Grassmann and Heyde (1929)	0·2	90% alcohol	0·004% t.p.	Clear blue	Clear blue	0·01 N alc. KOH
Two-indicator titrations—						
Henriques and Sørensen (1910)	20*	11% CH_2O	Litmus paper 0·0033% p.p.	6·8 (spot) —	— *ca.* 9·0	0·2 N NaOH
Löers (1920), Grünhut (1919)	20†	8% CH_2O	0·002% n.r. 0·0037% p.p.	7·1 —	— 9·2	0·1 N NaOH
Harris (1923)	5	80% alcohol	t.p. m.r.	Pale blue —	— 5·4	(0·1 N HCl)
Northrop (1926)	5	6% CH_2O	n.r. 0·004% p.p.	*ca.* 7 —	— 8·5	0·01 N NaOH
Tillmans and Kiesgen (1927)	5	85% alcohol	n.r. 0·0036% t.p.	7 —	— First blue	0·1 N NaOH

* —PO_4, —CO_2, and NH_3 free. † —PO_4 and —CO_2 free.

Owing to this diversity of method, it was desirable to compare the application of typical methods to complex material under identical conditions. These titrations were therefore conducted under the following standardized conditions: *Formaldehyde titration* from the following end-points in water to the same end-points in 9% CH_2O (9% at end of titration): (*a*) *ca.* p_H 7·5, mid-point of 0·001% n.r.; (*b*) p_H 8·4, first definite colour of 0·004% p.p.; (*c*) p_H 9·4, first definite colour of 0·01% t.p.; from these titres, the titre, n.r. (aq.) to t.p. (formaldehyde), was also determined. *Alcohol titration* from the p.p. and t.p. end-points in water to the same end-points in alcohol (85% at end of titration), and from the t.p. end-point in 85% alcohol to a m.r. end-point (first redness, p_H 6·0) also in 85% alcohol. *Formaldehyde-alcohol titration* from the p.p. end-point in water to the same end-point in 3% CH_2O-75% alcohol (% at end of titration). Indicator concentrations were maintained at 0·004% p.p. and t.p., and 0·0015% m.r. where not otherwise mentioned.

Thymolphthalein in the Formaldehyde Titration.—Sørensen (1907) was only able to use thymolphthalein in the presence of formaldehyde if at least 13% alcohol was also present. Foreman (1928) has discarded it entirely. From a further examination it is found that t.p. is slowly (10 minutes) but completely precipitated by formaldehyde from any aqueous solution not alkaline to p_H 11. The effect is reversed by heat, by concentrations of alcohol above 12%, or by organic solvents in general. Comparable precipitation by formaldehyde is not observed with thymol blue, phenolphthalein, or other phthalein or sulphonphthalein indicators. t.p. may give approximate results in the formaldehyde titration if the technique is sufficiently rapid.

Titration Results.—In Table X comparisons using these representative methods on a series of leaf extracts have been made. Great refinement was regarded as superfluous, since no titrimetric method can be specific and yet quantitative for amino-N. The titrations were carried out as described with ordinary titrimetric care. As an example, full data for a runner bean extract are given. 25 c.c. of the coloured extract, containing 42 mg. total N, and 12·5 mg. amino-N (Van Slyke), were brought to p_H 5·8 and decolourized by addition of 4 c.c. saturated lead acetate, etc. After removal of lead, and washing, 33 c.c. of very pale yellow extract were obtained. 2 c.c. samples of this extract were taken and estimated. Details are given in Table IX.

The end-points with n.r. and m.r. were very poor, as the divergence in duplicate titrations shows, while data with t.p. are at best only approximate. For these reasons, titrations other than those to p.p., are quoted in Table X to the nearest 5%. Titrations with broad bean were always obscured by the appearance of brown colours in alkaline solutions.

Nevertheless, certain main features of the comparisons are quite outstanding. For instance, the *formaldehyde titration* with t.p. as an indicator is consistently little more than half as large as with p.p. as indicator. With n.r., the titration is about 90% as large. With the two-indicator titration, n.r.-t.p., titrations were about 120% as large as the p.p. titration. The results did not show much fortuitous variation due to phosphates, etc., as these had been precipitated as lead salts. The accuracy of the latter estimation is

Table IX.—Details of amino-N determination in runner bean extract.

Van Slyke estimation: 1·04 c.c. nitrogen (corrected for blank) at 17° and 760 mm. = 0·60 mg. amino-N per 2 c.c.

Indicator.	Aqueous titre (c.c. N NaOH).	Titre increase.	Reagent blank.	Corrected increase.	Mg. amino-N per 2 c.c.	% amino-N (Van Slyke).
Formaldehyde titration. 2 c.c. 40% formaldehyde per 2 c.c. aliquot.						
		c.c. 1·01 N/35 NaOH.				
n.r.	*ca.* 0·28	2·30 2·40	1·3	1·05	0·42	70
p.p.	*ca.* 0·29	2·60 2·62	1·4	1·21	0·49	82
t.p.	*ca.* 0·31	2·20 2·25 (rapid)	1·6	0·62	0·25	42
n.r.-t.p.	—	3·05 3·10 (rapid)	1·6	1·47	0·60	100
Alcohol titration 25 c.c. 95% alcohol per 2 c.c. aliquot.						
		c.c. 0·051 N alcoholic NaOH.				
p.p.	*ca.* 0·29	1·42 1·45	0·17	1·27	0·91	152
t.p.	*ca.* 0·31	1·05 1·10	0·22	0·85	0·61	102
		c.c. 0·049 N alcoholic HCl.				
t.p.-m.r.	—	1·7 1·8	0·22	1·53	1·05	175
Formaldehyde-alcohol titration. 20 c.c. 95% alcohol per 2 c.c. aliquot.						
		c.c. 0·051 N alcoholic NaOH.				
p.p.	*ca.* 0·29	1·35 1·35	0·13	1·22	0·87	145
20 c.c. 95% alcohol + 2 c.c. 40% formaldehyde per 2 c.c. aliquot.						
p.p.	*ca.* 0·29	2·25 2·30	1·05	1·22	0·87	145

reduced, however, by the difficulty of the orange and greenish end-points. If sufficient material is available, this titration is more conveniently performed on aliquots, the first to n.r. in water, the second to t.p. (p.p.) in formaldehyde. The formaldehyde (p.p.) titration estimates in decoloured grass extracts about 95% of their amino-N (Van Slyke), in brussels sprouts 85%, and in legumes, variable but generally 70 to 85%. Whether the individual variations are due to errors of the Van Slyke or the formaldehyde method has not been ascertained, but the evidence is undoubtedly that general magnitudes by the Van Slyke method are reproduced by the formaldehyde (p.p.) method.

Table X.—Comparative determination of amino-N in leaf extracts. Titres calculated as % of amino-N (Van Slyke) of treated extracts.

Extract.	Amino-N (Van Slyke) (mg. per 2 c.c.).	*Formaldehyde titration.*				*Alcohol titration.*			Formaldehyde alcohol titration p.p.
		n.r.	p.p.	t.p.	n.r.-t.p.	p.p.	t.p.	t.p.-m.r.	
Cocksfoot, lead filtrate	0·98	80	96	55	110	159	105	180	168
Cocksfoot, Neuberg precipitate	0·96	85	91	65	115	108	50	100	115
Cocksfoot, Neuberg filtrate	0·10	—	*95*	—	—	*1000*	*1400*	*3000*	*1000*
Cocksfoot, norite filtrate, p_H 1·1	0·64	—	90	—	—	156	—	—	—
Cocksfoot, norite filtrate, p_H 2·6	0·62	—	101	—	—	152	—	—	—
Lucerne I, lead filtrate	0·38	—	85	—	—	260	—	—	255
Lucerne II, lead filtrate	0·52	—	71	50	125	350	360	405	350
Lucerne III, lead filtrate	0·99	—	73	—	—	141	—	—	—
Vickery's "alfalfa filtrate," lead filtrate	1·40	—	103	—	—	163	—	—	—
Broad bean I, lead filtrate	1·59	70	82	40	105	98	15	105	103
Broad bean II, lead filtrate	0·64	—	75	—	—	154	—	—	—
Broad bean III, lead filtrate	0·72	—	91	—	—	151	30	155	151
Runner bean, lead filtrate	0·60	70	82	40	100	150	100	175	145
Brussels sprouts, lead filtrate	0·82	90	86	45	110	182	150	215	187
Na malate (% 1 equivalent)	—	—	—	—	—	—	—	20 (60)	—
Na succinate (% 1 equivalent)	—	—	—	—	—	—	—	50 (95)	—

This is not true of titrations using large concentrations of alcohol. In the *alcohol and the formaldehyde-alcohol titrations* analyses varying fortuitously from the Van Slyke figures by 100 to 350% are obtained. The belief that these variations are due to magnesium is still further strengthened by their nature, firstly because they are not removed by norite or lead acetate and are not precipitated by Neuberg's reagent, but are greatly magnified in the filtrate therefrom (the low amino-N (Van Slyke) of this filtrate makes the percentage figures approximate only); secondly, because they vary markedly in different extracts of the same plant, and thirdly, because, where they are largest, the titration to t.p. is even larger. This is not true of extracts in which the alcohol titration to p.p. agrees with the Van Slyke figure, but is behaviour characteristic of solutions of magnesium. In the absence of magnesium, the t.p. and t.p.-m.r. titrations are analogous to the similar titrations in formaldehyde. The t.p.-m.r. end-points are difficult, as expected, and it is shown, for example (Table X), that in the back titration to m.r., the weak groups of malic and succinic acids were 20 and 50% estimated, respectively, or, using the orange end-point of Harris (1923), 60 and 95% estimated. Succinic acid was not removable by lead acetate at p_H 6.

As it is unfortunately impracticable to remove magnesium from the extracts, the alcohol and formaldehyde-alcohol methods (which yield closely similar results throughout) must be discarded in most biological analyses.

Titration by Acetone Addition.—The substitution of acetone for alcohol in the titrations was introduced by Foreman (1920). In this solvent, however, interference by magnesium will be even more pronounced. In general, any characteristic of the alcohol titration reappears in the acetone titration with greater emphasis, and the method has not been separately examined.

Titration by other Aldehydes.—0·1 M glycine and 0·04 M Na aspartate gave no increase of phenolphthalein titre in 0·5 M chloral hydrate or M glucose in the cold, but gave a slow increase with 2/3 saturated furfural, with colouration, on standing at about p_H 9.

Discussion.

No critical biological application of the methods for determining amino-N in the light of recent developments in theoretical interpretation has so far appeared. Methods, preferred in one field for empirical reasons, have been adopted without discrimination in other fields as having the same degree of accuracy. For instance, the alcohol titration method, long used with satisfaction in following enzymic reaction, has recently been applied both to meat extracts (Foreman, 1928) and to plant extracts (Rahn, 1932). By the former a difference between alcohol titratable and formaldehyde titratable groups is called "non-volatile amines," and by the latter a difference between amino-N (alcohol) and amino-N (Van Slyke) is called "firmly-bound polypeptides." No such assertions would have appeared if the theoretical implications had been more clearly visible at the time. It is now possible to forecast more correctly the value of any method, and to choose the one most appropriate for any particular purpose.

Van Slyke Method.—The limitations of the Van Slyke method have been sufficiently well investigated experimentally to need little further discussion (Van Slyke, 1911; Schmidt, 1929). Errors due to manipulation (Lamson, 1924; Sørensen and Walther, 1928), or the presence of tannins (Rahn, 1932) have been variously considered. The imperfections among actual α-amino acids and peptides were mentioned in the introduction. Related groups such as amino groups not in the α position to —COOH have been examined by Dunn and Schmidt (1922), or in peptides by Greenstein (1933). Of other substances, reaction has been demonstrated with ammonia and the amines (Van Slyke, 1912; Rosenthaler, 1929), urea (Plimmer, 1925), amides, ureides, and guanidines (Wilson, 1923; Plimmer, 1925), glutamine amide-N (Chibnall and Westall, 1932), indoles (Schmidt, 1929), and amino-purines and -pyrimidines (Wilson, 1923). No reaction is observed with proline, oxyproline (Van Slyke, 1911), betaine, sarcosine, brucine (Wilson, 1923), hydrazines, certain cyclic nitrogen compounds (Rosenthaler, 1929), glucose, oxalic, formic, and glycollic acids (Schmidt, 1929). Often the temperature coefficient of the rate of reaction is large (Dunn and Schmidt, 1922; Wilson, 1923). Under our conditions (17° and 10 minutes) the reaction has probably estimated, glutamine 185%, lysine 90%, lysyl peptides 100%, glycyl peptides 130%, cystine and glycine 107%, and adenine 25% of the expected amino-N, and ammonia 20%, creatinine 20%, and urea, ureides, and guanidines 0 to 5%, of one N atom. The method of Van Slyke is therefore not free from considerable uncertainty where, as in plant extracts, many of these substances may be present.

Titration Methods, Theoretical.—The limitations of the titration methods require fuller discussion as, although the methods are themselves varied, they may be treated comprehensively instead of empirically for each method as hitherto. From the theory of acid-base titrations (see *e.g.*, Cole, 1928, 12 *et seq.*) it is an inevitable consequence that gradual titration from 1% neutralization to 99% neutralization requires a change of 4 p_H units. Conversely, if some group is being titrated from an initial point (perhaps an indicator) to another indicator whose colour change is only 4 p_H units removed from the first, titration cannot exceed, and may be less than 98% theoretical. The 98% titre is a maximum that can only be obtained if the p_H of 50% neutralisation ($p_{K'}$) of the group is midway between the indicators, *i.e.*, when, say, KH_2PO_4, $p_{K'}$ (second phosphoric) = 7·2, is titrated from m.r., p_H 5·2, to t.p., p_H 9·2. If instead the $p_{K'}$ is 3 p_H units from the first indicator and only one from the second or *vice versa*, *i.e.*, titrating KH_2PO_4 from bromphenol blue (4·2) to p.p. (8.2), the first indicator colour (3 units away) occurs at

0·1% neutralization, the second (1 unit away) when there is still 9·1% unneutralized. Titration cannot exceed 90·8% theoretical, and the second end-point can never be sharp.

Consequently, in any titration when for any reason the p_H range between end-points is limited, it is essential to choose indicators such that the group titrated stands midway between them. Or if a number of groups of similar strengths, in a complex medium, are to be estimated together, their mean $p_{K'}$ should stand midway between the indicators. Or, finally, if the mean $p_{K'}$ is unknown, one can at least state that titration between indicators which lie too acid or too alkaline will be smaller than titrations between the best situated indicators. These general consequences of titrimetric theory are, of course, well known.

Now, as described in the previous paper (p. 129) the effect of adding alcohol or formaldehyde to solutions of amino acids is to reverse the positions which the indicator and amino groups occupy with respect to each other. Thus, whereas in water p.p. becomes coloured at p_H 8·2, *i.e.*, about 1 to 1·4 p_H units on the acid side of the $p_{K'}$s of most amino acids, after addition of formaldehyde or alcohol, p.p. will not become coloured until 1 to 2 units on the alkaline side of their $p_{K'}$s.* In other words, whereas p.p. is coloured in water before the amino groups have been titrated very far with NaOH, in formaldehyde or alcohol the amino groups must now be very considerably titrated before p.p. becomes coloured. This gives an increased titration, after addition of the formaldehyde or alcohol, which is a measure of the amino-N content of the solution.

The effect may be quite legitimately compared with a simple aqueous titration between two indicators, in which the p_H range between the indicators is for 10% formaldehyde 2·5 to 3 p_H units and for 85% alcohol 3·5 p_H units. This p_H range is definitely too small for quantitative titration of amino groups by the one-indicator method. Moreover, the maximum titre, 90 to 96% will be greatly influenced by the choice of indicator, which should lie as far to the acid side of the mean amino nitrogen $p_{K'}$ before formaldehyde or alcohol addition as it does to the alkaline side afterwards. Since, therefore, the first colour of p.p. appears at 1 to 1·4 p_H units (half the available titration range) on the acid side of the mean $p_{K'}$ of amino groups, *ca.* 9·6, this indicator seems, very happily, to be an ideal choice for the titration.

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* The term $p_{K'}$ is here applied to a base in the sense suggested by Brønsted, *i.e.*, $= 14 - p_{K'_b}$, so that it is expressed directly in terms of the p_H scale. All p_K·s quoted are the accepted values from recent literature.

One-indicator Methods.—Thus no one-indicator method is able to provide fully quantitative measurement of amino-N. The percentage estimation, which can be easily calculated, will vary for the separate amino acids according to the $p_{K'}$ of the individual amino acid and the p_H of the chosen end-point (for p.p. (p_H 8·2) the ideal $p_{K'}$ is *ca.* 9·7). Strong basic groups (betaines and the guanidine groups of arginine and creatine) are not estimated; neither are weak basic groups (the cyclic N of histidine, $p_{K'}$ 6·0, creatinine, 4·7, and tryptophane, $<$ 1—compare Sørensen, 1907). Amines (and amino groups not α to —COOH), mean $p_{K'}$ 10·6, proline, 10·6, and sarcosine, 10·0, will be incompletely titrated at the final p.p. end-point (compare Sørensen, 1907; Clementi, 1915; Martens, 1927) and would give more quantitative results with a more alkaline end-point. On the other hand, the amino groups of phenylalanine, tyrosine, histidine, 9·2, lysine (first), arginine, 9·0, and cystine, 7·5 and 9·0, and particularly of any peptides, 8·3, will be partially titrated before the first end-point (compare Henriques and Gjaldbäk, 1911; Foreman, 1920; Willstätter and Waldschmidt-Leitz, 1921; Martens, 1927), and would be better estimated with a more acid end-point. Incidentally, the titre ratios, litmus to p.p. (8·3) in water: litmus to p.p. (9·1) in formaldehyde, quoted by Henriques and Gjalbäk (1911) and Grünhut (1919), give very useful indication for many examples of the extent of initial titration. Some less common amino acids requiring special notice are, thiol-histidine 8·5, cysteine, 8·2, di-iodotyrosine 7·8. In the light of this discussion, the result of Table X that n.r. (p_H 7·5) estimates 90% of the p.p. titre (p_H 8·4) of our extracts, whereas t.p. (p_H 9·4) estimates only 50% is not at all surprising.

Of the one-indicator methods, the formaldehyde titre has a decided advantage over the alcohol titre, in that it depends on a chemical reaction apparently specific for amino groups, whereas the alcohol titre depends on a physical phenomenon without chemical specificity. Thus formaldehyde titration estimates, as above, only primary amines, secondary amines (proline and sarcosine), and ammonia ($p_{K'}$ 9·3—compare Sørensen, 1907); whereas alcohol titration includes to some extent any group of $p_{K'}$ 9 to 10 whose strength is altered differently by alcohol from the alteration of strength of p.p. (a multivalent acid). Thus, alcohol titration (p.p.) will partly estimate not only the above bases, but also magnesium or such alkaloids as have appropriate basicity (*e.g.*, nicotine, $p_{K'}$ 8·1, will be part titrated) and even monobasic acids of similar $p_{K'}$s. This has been clearly shown in Table VI for weak acids, and in Table VII for magnesium. Again, alcohol titration of extracts containing much inorganic matter or protein is complicated by precipitation of material, with possible loss of amino-N (Sørensen and Walther, 1928). For both these reasons, the general usefulness of the alcohol titration as compared with the formaldehyde titration is seriously limited.

Two-indicator Methods.—These methods, in effect, titrate first in aqueous solution from n.r. (p_H 7·0) to p.p. (p_H 8·3), covering 1·3 p_H units, and then to p.p. again after formaldehyde or alcohol addition, giving 2 to 3 p_H units more. This has the advantage (*a*) of a total p_H range of 4 units (2·5 units to the mean amino-N $p_{K'}$ and 1·5 afterwards) permitting 97% titration, and (*b*) of much completer estimation of those amino acids with weak —NH_2 groups (arginine, lysine, cystine, and the dipeptides—see below). On the other hand, as has been noted, it is dangerously interfered with by phosphates, $p_{K'}$ 7·2, and by weak acids such as chlorogenic acid, 8·5, silicic acid, 9, or the second groups of citraconic and maleic acids, 6·6, if these should be present in the extract under examination. Harris' two-indicator method using m.r., has been shown to be disturbed not only by these acids, but even by the weak groups of malic, 5·1, succinic, 5·6, or citric acids, 5·5.

Therefore, though these two-indicator methods give greater accuracy in estimating solutions which contain solely amino acids and peptides, they provide reasonable accuracy in other solutions only if pre-treatment has removed all interfering groups somewhat titratable in water between the chosen indicators. Our very tentative results show that decolouration by lead acetate has precipitated sufficient of these groups to allow a close correspondence between the one-indicator and the two-indicator titres, the latter being larger by 20%. In such case, however, the two-indicator methods still suffer from the original specificity defects of the one-indicator methods.

It is, of course, obvious that the two-indicator *aqueous* titration methods of Harris (1923), Tillmans and Kiesgen (1927), and Felix and Muller (1927), titrating from p_H 7 to p_H 11·6, can only be applied safely to solutions comprised solely of amino acids and peptides as titratable material.

Previous Investigations.—It remains, lastly, to summarize the few previous comparative investigations. Cook (1914) compares the Van Slyke and formaldehyde methods, used without description, on twelve commercial vegetable and meat extracts, and finds the formaldehyde titration to average but 60% of its Van Slyke equivalent. That this may be due to some peculiarity of the extracts such as a high proportion of peptide-N is suggested by the later work of Lüers (1920) on two protein digests and one plant extract, in which amino-N by his two-indicator formaldehyde titration is 107 to 125% of amino-N (Van Slyke). In Table X our results are of this latter order. It is recalled also that the Van Slyke method has been variously criticized owing to the possibility of reaction with reducing agents present, or of hydrolysis by action of the strong reagents employed. Rahn (1932) comparing Van Slyke and alcohol (p.p.) values on a root extract, finds the latter value to be in excess, almost certainly an indication of magnesium, but described by this author as "firmly bound polypeptides."

Purely titrimetric methods have been compared by Tillmans and Kiesgen (1927), who obtain substantial agreement upon seven food extracts with their two-indicator methods: n.r. to p.p. in formaldehyde, n.r. to t.p. in alcohol, and n.r. to tropaeolin 0 in water. Since each method uses the same initial point, weak acids may be included in these estimations unnoticed unless so weak as to be titrated by tropaeolin 0 but not by p.p. One meat extract gives 108% of its p.p. value for tropaeolin 0, or 105% for t.p. This may be due to such a weak acid, or to magnesium. Agreement between contrasted two-indicator titrations does not necessarily imply accuracy and specificity in the estimation of amino-N. Foreman (1928) has compared the formaldehyde and alcohol titrations as one-indicator methods, using meat extract media and one sample of urine. He finds the alcohol titre, after distilling off ammonia (volatile bases), to be invariably higher than the formaldehyde titre. The disparity, regarded by him as due to "non-volatile amines," is almost certainly due to the partial inclusion of magnesium in the alcohol titration. Thus, a normal 24-hour urine contains amino-N and magnesium in quantities averaging (in terms of titratable "N") 25 and 20% of ammonia-N; it is rather significant that the formaldehyde titre of the urine and its actual disparity from the alcohol titre were respectively 14% and 18% of the ammonia titre in Foreman's experiment. Likewise, the disparity in the meat extract media, which was also large, showed behaviour expected of magnesium, since it remained constant during the course of bacterial action, and was precipitable from neutral solution to the extent of 10 to 25% by adding 88% alcohol.

Foreman (1920), Harris (1923), and Martens (1927) compare various titrimetric methods upon pure amino acids. The results accord with the expectations from the known p_Ks of the amino acids, and the influence on them of alcohol or formaldehyde addition. The comparison of methods upon protein hydrolysates is discussed in the previous paper (p. 138).

Reasons for neglecting the Folin colourimetric method appear in papers of Ellinghaus (1925) on protein digests, and of Zirm and Benedict (1931) on protein-free blood sera. These criticisms, however, require revaluating in the light of the recently improved method of Danielson (1933). The method is preferred to the Van Slyke method for bacterial culture media by De Bord (1923).

Conclusions.

In a simple amino acid-polypeptide solution, two-indicator titrations are obviously most nearly quantitative. For the same end-points, acetone (NaOH) titration is more nearly quantitative, while alcohol (NaOH) titration is slightly more so than formaldehyde (NaOH) titration. The first two methods are discounted, however, by the common occurrence of precipitation, the latter by the inability to use thymolphthalein as indicator. Acetone (HCl) titration is not a direct measure of amino-N, as it estimates —COOH groups.

In coloured biological extracts, the Van Slyke method is preferred in spite of its uncertainties, since effective decolourants for titration removed also 15 to 25% of amino-N (Van Slyke) by their action. In the extracts examined, duplicate Van Slyke estimations did not vary by more than 0·03 c.c. nitrogen. If decolourants may be used, the consequent loss of N being purposely neglected, then lead acetate at p_H 6 is preferred, as it removed rather less N than did norite, and simultaneously precipitated weak acids disturbing the titrations (phosphates, chlorogenates, etc.).

In lead-treated biological extracts, the two-indicator formaldehyde titration (n.r.-p.p.) is preferred (Lüers, 1920; Grünhut, 1919), provided it is compared always with the one-indicator formaldehyde titration (p.p.) as a control of the fortuitous presence of disturbing titratable groups. As far as present information is available, it is less subject to chance variation than is the Van Slyke method. Alcohol and aqueous (NaOH) titrations are to be avoided, as they have no real specificity for amino-N.

In non-coloured biological extracts, the remarks for lead-treated extracts apply, except that two-indicator methods are not recommended unless weak acids are first removed by some method such as the lead precipitation above. For direct estimation, the one-indicator formaldehyde titration (p.p.) is preferred, on the whole, to the Van Slyke method, as being quicker and more specific for amino-N, but it is probably not more than 90% quantitative and would be considerably less so in the presence of much peptide amino-N.

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Summary.

Comparison of titrimetric and Van Slyke methods of estimating amino-N in biological extracts has been made. Eight modifications of the formaldehyde and alcohol titration methods are included in an examination of plant leaf extracts.

All alcohol (or acetone) methods for amino-N are discarded for biological extracts owing to lack of specificity. Magnesium, which cannot be readily removed, and various weak acids, are titrated as amino-N by such methods.

Existing literature on the estimation of amino-N is summarized and the general usefulness and accuracy of the methods discussed. For coloured biological extracts, the Van Slyke method is preferred, but the formaldehyde (phenolphthalein) titration has advantages for colourless extracts. The implications of the zwitterion conception on the interpretation of titrimetric methods is stressed.

The effect of p_H on the efficiency of norite and lead acetate as decolourants of leaf extracts has been investigated. Lead acetate at p_H 6 is adopted in preference to norite at p_H 1. The colourstuff of the extract is found to migrate as an ampholyte during electrodialysis.

Note.—In a paper which has recently appeared (October, 1933), Van Slyke and Kirk examine the Van Slyke, Folin colorimetric, and formaldehyde methods as applied to blood and urine. They obtain substantial agreement between the Van Slyke and formaldehyde methods, and severely criticize the colorimetric method applied as originally described. They examine the acetone titration, adapted to blood sera by Zirm and Benedict (1931), and show it to titrate carboxylic acids.

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The Differential Titration of Mixed Strong and Weak Acids. A Method for "Free HCl*" in Gastric Contents, etc.*

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It is frequently convenient to be able to distinguish between strong inorganic acids and weaker acids present in the same aqueous solution. Biochemically, such a distinction is required in analysing gastric contents for "free HCl," in titrating blood for "organic acids," in determining free acidity during the course of peptic digestion, and so on. Chemically, it is useful to be able to estimate by direct acid titration moderately strong acid groups combined as salts (such as sodium acetate, sodium potassium tartrate, potassium citrate, lead acetate, etc.). Available methods were either entirely approximate or even positively inaccurate. To remedy this position, a simple modification affording considerable quantitative improvement is now proposed.

The difficulty common to current methods (see Hawk, 1931; Hollander, 1931) is chiefly that of distinguishing strong acids (fully dissociated) from other unknown acids only slightly less strong (not fully dissociated), by an end-point which depends on p_H alone. No new choice of indicator can improve the quantitative uncertainty as to how far these unknown moderately strong acids have participated as "strong acids" in an estimation, and, in fact, no improvement is possible unless the acidity of the moderately strong acids is so depressed as to minimize the extent of their interference. The inherent advantage of titration in non-aqueous solvents over ordinary aqueous titrations in this direction has long been realized (Bishop, Kittredge, and Hildebrand, 1922); but the procedure cannot be used in biochemical estimations, where the experimental material is in aqueous solution. Yet it is strange that the obvious device —the basis of which was established by Cray and Westrip (1925)—of adding miscible organic solvents (acetone) to these aqueous solutions, has not been adequately exploited as a simple practical method. Preliminary notice of such adaptation has already been given (Richardson, 1932), but detailed presentation has been delayed to permit treatment of other aspects of mixed solvent titration (p. 121).

Current Methods.

Current methods depend largely upon the empirical selection of optimum conditions for titration of any particular fluid. In the special case of gastric and blood analyses, these methods are of four types: (1) titration of a secondary product liberated by the action of free hydrions (iodine from Sahli's reagent); (2) acid-base titration to a chosen p_H end-point—thymol blue p_H 2, tropaeolin 00 p_H 2·7 (Palmer, 1926), Töpfer's indicator (salmon pink) p_H 3·5, bromphenol blue p_H 3·5 (Hollander, 1931), and other indicators; or electrometrically p_H 2·3 (Perlzweig and Delrue, 1928)—of these, Töpfer's indicator is traditionally used, the others being advocated quite recently; (3) determination of p_H, often colorimetrically, and calculation of free acidity corresponding thereto; (4) progressive neutralization until a spot reaction characteristic of "free HCl" cannot be further observed (red colour on evaporating with Gunzberg's reagent). These methods are commonly considered to be unsatisfactory, being used simply in lieu of better alternatives (Christiansen, 1912; Hollander, 1931). The fault is that each method is susceptible to hydrions from any source if in sufficient concentration, and that many acids are strong enough to provide such concentrations.

Sahli's Reagent.—This reagent is a mixture of equal volumes of 8% KIO_3 and 48% KI, 1 c.c. of which is added to 2 c.c. of gastric contents + 10 c.c. of water, and the liberated iodine titrated with very dilute sodium thiosulphate after 5 minutes standing. An elementary knowledge of the oxidation-reduction potentials of iodine and KIO_3, however, shows that iodine will be quantitatively obtainable from the latter at any p_H acid to p_H 6·5, and that the reagent is therefore sensitive to all ordinary organic acids. This is confirmed in Table I, though it is found that acid groups having buffering power about p_H 5 to 7 react only slowly, *i.e.*, that 5-minutes reaction reduces acidity only to p_H 5·5.

Indicator Titrations.—The end-points for these titrations are inevitably difficult and vague (see also Rehburg, 1925). Applying first principles (p. 123) to the titration of the stronger acid groups, we find that one volume of, say, 0·1 N Na lactate ($p_{K'}$ 3·9) would not be 99% acidified until p_H 1·9, when additional 0·1 N HCl (x) sufficient to render the total volume ($= 1 + 0{\cdot}99 + x$ vols.) 0·013 N with respect to "free HCl" (neglecting activity correction) would be required; this amounts to 29% extra 0·1 N HCl. Titrating then to the finest end-point visually distinguishable (0·1 p_H units minimum), and accepting p_H 2·0 to 1·9 as this end-point, even this small range permits variation from 121% (99 + 22) to 128% (99 + 29) HCl addition by reason of the "free HCl" required. Thus not only do these titres require extensive correction for "free HCl," but the visual end-points are themselves uncertain to the extent of at least 7%; were account to be taken of activity corrections, the uncertainties would be more pronounced. For stronger acid groups, such as pyruvic acid (2·6), amino acids (2·6 to 1·8), phosphoric acid (2.1), the difficulty of titration is accentuated in corresponding degree. Thus the common observation that visual end-points are more prolonged the stronger the acid groups titrated is definitely the fault of the acid end-point, not of the individual defects of the indicator.

Since this is so, there has been a tendency, for the sake of sharper titration, to adopt less acid end-points than should be used and arbitrarily to assume that titratable acidity acid to the end-point represents "free HCl," or alkaline, "organic acids." The assumption is only justifiable when strong organic (and phosphoric) acids are absent, as in phosphate-free urines (p_H 2·7, Palmer, 1926) or pure gastric juice (p_H 3·5, Hollander, 1931) when a small correction for "free HCl" at the chosen end-point is always applied. Otherwise, it becomes merely an attempt to approximate a sort of "equivalence-point" at which the "free HCl" is fortuitously equivalent in amount to the untitrated salts of "organic acids." This arbitrary compensation of the excess HCl required as "free HCl" at the chosen end-point, against the defect HCl due to incomplete acidification of rather strong groups, varies in effectiveness according to the concentration and acid strength of the groups present. Consequently, no standard "equivalence-point" can be chosen which will suit all cases; nor can titration to any "equivalence-point" be at all sharply defined.

It should be clear from Table I that the p_H 2 end-point is better suited to exclusive titration than that at p_H 3·5. Thus, though with thymol blue only 60% of the "free HCl" of a gastric contents of about p_H 1·6 (0·03 M HCl) will be titrated, this is compensated by titration of 5 to 30% of amino acids ($p_{K'}$ 2·6 to 1·8), 40% of phosphoric acid, and perhaps small amounts of peptides, citric acid, etc. The net result is the desired one that acids other than HCl will seemingly be largely untitrated, though unfortunately end-points will be very poorly defined. With Töpfer's reagent, on the other hand, any improvement in end-point is discounted by the fact that "free HCl" titre must unintentionally include many other acids. A distinctive modification to improve end-point definition eliminates indicator methods entirely by using the quinhydrone electrode to demark a representative "equivalence-point" at p_H 2·3 (Perlzweig and Delrue, 1928). Yet even were determination of this "equivalence-point" free of the several possible objections, the absolute dependence of such titration as a measure of "free HCl" on the fortuitous presence or absence of moderately strong acids is a very serious limitation.

Determination of p_H.—From the determined p_H of a gastric contents it is theoretically possible to calculate the "free HCl" present, since moderately strong acids present do not contribute largely to the maintenance of such strong acidities. Yet as the p_H is often determined colorimetrically by standard indicators, uncertainty in matching shades, even though more vital to the success of the method, is inevitable; while electrometric methods are not only tedious but subject to error from uncertain junction potentials. At the same time, conversion of p_H into terms of "free HCl" involves considerable activity correction, which must vary from sample to sample with its salt content, on the one hand, or the influence of proteins, etc., on its dielectric constant, on the other. Hollander (1931) adduces reasons for discarding the method for gastric contents and all complex mixtures, although using it for pure gastric juice. The method is so systematically uncertain that its continued use is sufficient commentary on the inadequacy of alternative methods.

Table I.—The selectivity of current methods for strong acids. Data as % one acid group. $p_{K'}$ values from recent literature.

Acid.	$p_{K'_1}$.	$p_{K'_2}$.	$p_{K'_3}$.	Sahli's reagent.		Töpfer's reagent p_H 3·5.	Gunzberg reagent.	Thymol blue p_H 2.
				5 mins.	270 mins.			
0·1 M HCl	—	—	—	100	—	99	99	76–81
0·03 M HCl	—	—	—	—	—	—	—	52–60
0·1 M HNO_3	—	—	—	100	—	99	99	77–82
0·05 M H_2SO_4	—	1·6	-	100	—	98	185–190	133–140
0·00025 M HCl, HNO_3, or H_2SO_4	—	—	—	—	—	—	+	—
0·05 M oxalic acid	1·2	4·1	—	200	—	120	85–90	53–63
0·1 M phosphoric acid	2·0	6·8	11·9	109	118	95	25–35	35–43
0·05 M glutamic acid HCl	2·2	4·2	9·6	200	—	105	16–18	10–17
0·1 M glycine HCl	2·4	9·6	—	100	—	91	7–11	7–14
0·03 M phthalic acid	2·9	5·4	—	187	191	80	—	0
0·05 M citric acid	3·0	4·6	5·8	234	274	88	+	0
0·05 M tartaric acid	3·0	4·5	—	197	—	76	+	—
0·05 M malic acid	3·4	5·1	—	186	193	50	?	—
0·1 M lactic acid	3·9	—	—	99·5	—	38	—	—
0·05 M succinic acid	4·2	5·4	—	157	191	15	—	—
0·2 M acetic acid	4·7	—	—	85	96	5	—	—
Saturated CO_2	6·3	—	—	18	31	—	—	—
0·2 M boric acid	9·2	—	—	0·3	—	—	—	—
0·05 M KHPhthalate	—	—	—	82	94	—	—	—
0·2 M KH_2PO_4	—	—	—	8	21	—	—	—

Gunzberg's Reagent.—This reagent is a solution of 1 part vanillin and 2 parts phloroglucinol, which, evaporated to dryness, gives a characteristic red colour on evaporating with a spot of acid. By progressive neutralization, the end-point at which further colour is no longer produced is determined. Colour production is more sensitive if p_H is maintained by dilute acids rather than by buffer mixtures (see also Christiansen, 1912). This is presumably because evaporation considerably decreases the p_H of dilute acid solutions by concentrating the acid, but does not greatly alter that of buffer solutions. Thus the fact that 0·00025 N HCl, H_2SO_4, and HNO_3 (p_H 3·6) and 0·001 M H_3PO_4 (p_H 3·05) are all sensitive to the reagent, whereas 0·1 M H_3PO_4 as a buffer is almost insensitive at 30% neutralization (p_H 1·7), is presumably due to the development just before dryness of a p_H of about 1·6 in each case (assuming one hundredfold increase of concentration). The observation that colour is given in 0·1 N solution only by acids stronger than $p_{K'}$ 3·5 (except phthalic acid) is of corresponding implication. Whatever the interpretation, in practice strong acids may be very largely titrated by NaOH with this end-point, whereas most moderately strong acids lose their sensitivity to the reagent with comparatively small NaOH additions. Thus the method, although tedious, is a useful improvement on methods depending on differentiation by a p_H end-point alone.

Conclusion.—The conclusion is plain that current methods, though providing tentative and empirical bases for comparison, do not represent with any reality the actual " free HCl " of the fluids investigated. Gunzberg's method or a titration at p_H 2 provide most nearly a satisfactory differentiation between " free HCl " and " organic acids."

Proposed Method.

The difficulty of differential titration hitherto has rested in the lack of sharp differentiation in acid strength between the strong acids and the moderately strong acids of the solution. Fortunately this particular differentiation is not equally impossible in aqueous acetone solution. The nature of acidity in relation to mixed solvent titration has been discussed elsewhere (p. 124). It will suffice to assert here that, whereas moderately strong acids are weakened 10,000 to 100,000 times (4 to 5 p_H units) by addition of 90% acetone, true strong acids are not greatly affected. This marked relative alteration of strength in 90% acetone makes it possible to titrate strong inorganic acids in the presence of weak and moderately strong acids, or *vice versa* without appreciable overlap.

Thus, considering the simple acidification of lactates ($p_{K'}$ 3·9), shown earlier to permit 7% uncertainty in the aqueous titration to p_H 2 to 1·9 (thymol blue), we find that in 90% acetone, titration is analogous to an aqueous titration of a group of $p_{K'}$ about 8. The result is that 99% titration by HCl can now be accomplished at p_H 6, necessitating only 0·002% extra "free HCl" instead of 29% (assuming original 0·1 N solutions). Likewise, it is even possible to titrate sodium oxalate ($p_{K'}$ 1·2, shifted to about 6) with HCl with little worse definition than that of sodium bicarbonate in water ($p_{K'}$ 6·3) using an indicator of p_H range about 3·5 in acetone. The considerable advance in sharpness of end-points and absence of "free HCl" correction that is attainable needs no stressing.

For differentiating the strongest acids, indicators for the p_H range 3·5 to 4·0 in 90% acetone are required, and are obtainable by using either a basic indicator of aqueous range about p_H 5·0, or an acid indicator of aqueous range p_H 2. By examination of numerous indicators (Table II), *p*-benzenesulphonic acid-azo-α-naphthylamine (called herein naphthylamine orange, by analogy with naphthol orange, methyl orange, etc.) has been selected, but erythrosin (iodeosin) is also suitable. Titration to two end-points is recommended, defined by the indicator colours in 90% acetone, (I) of 0·0004 N HCl, p_H 3·4 (salmon pink), and (II) of equal volumes of 0·05 M pyridine and pyridine HCl (orange yellow—approximately the same p_H as 0·0001 N HCl in 90% acetone = p_H 4·0). The purpose of the two end-points is to discern the extent of overlapping. Thus for most organic acids, end-point II will completely differentiate them from HCl, when the titre from end-point II to end-point I is due to HCl only, and should be the same as an appropriate blank on water or dilute NaCl (activity effects are large enough in 90% acetone to make the rough duplication of the salt concentration of the unknown a necessity in the blank). Where larger titres than this are observed, rather strong acids must be present and end-point I (p_H 3·4) is then more reliable (Table III). Titres either to end-points I or II are then corrected for their respective blanks (water blanks correspond to a concentration of 0·0004 N or 0·0001 N HCl in the acetone solutions, but 0·1 N NaCl blanks are almost double). As titrating agent, 0·05 M NaOH or HCl in 90% *alcoholic* solution is run in from a micro-burette suitably protected from CO_2, acetone being too volatile for convenient use in standard solutions. Indicator is used in the proportion of 1 drop of 0·1% alcoholic solution (75%) per 5 c.c. of solvent.

In this way, practically exclusive titration of true strong acids in the experimental solution may be accomplished, together with considerable improvement in end-point definition; for example, HCl, $HClO_4$, HNO_3, and $RHSO_4$ as strong acids may be suitably differentiated from H_3PO_4 and all organic acids as moderately strong or weak acids. The chief difficulty resides in the fact that the second ionization of sulphuric acid in 90% acetone is intermediate in acid strength between the limiting categories and obscures the end-point. Fortunately, however, salts of this one common acid capable of interference are quite easily removable by precipitation. The method on the whole, then, is simple, direct, and rapid, and applicable to any solution to which ordinary indicator methods apply. Biochemically, it provides with reasonable validity the important differentiation of "free HCl" from "organic acids," though its values do not purport to be the same as those from the vague and arbitrary "equivalence-points" of aqueous titration.

Linderstrøm-Lang's Acetone Titration.—The first application of the principle of the above differentiation was Linderstrøm-Lang's titration (1928) of "amino-N," actually a titration of the *carboxylic* groups of amino acids (p_K, about 2) by weakening their acidity in 90% acetone. The method was developed for its application to protein chemistry with the unfortunate result that it was considered "eine Methode zur Bestimmung von Aminostickstoff" (p. 48). This conclusion arises from a situation peculiar to the hydrolysis of proteins (p. 135), and although Linderstrøm-Lang once mentions that "die Wasserstoffionenanlagerung in vielen Fällen an der COO^- -Gruppe stattfindet" (p. 42), he nevertheless adopts the opposite interpretation throughout. His method must be considered a special case of the above general procedure.

Experimental.

Solutions of the acids used were standardized against CO_2-free 0·1 M aqueous NaOH with phenolphthalein (phosphoric acid with bromcresol green, amino-acids with bromthymol blue). On the basis of these titres, results are expressed as % of single acid groups, or in mixtures as % free HCl. Since general conclusions were all that was sought, materials were ordinary commercial preparations of the acids. Quoted results are the mean of comparable duplicates.

Sahli's titration was carried out on 2 c.c. aliquots with 0·02 N $Na_2S_2O_3$ and the usual starch end-point, over reaction times of 5 and 270 minutes. CO_2 absorption as a result of long standing was a negligible factor. Thymol blue and Töpfer's indicator titrations were carried out in 5 c.c. aliquots with 0·1 M NaOH and matched indicator end-points. Colour standards for the former indicator were prepared in p_H 2 HCl-KCl buffer and in p_H 2·3 phthalate buffer; for the latter a methyl orange colour in p_H 3·7 phthalate, corresponding to the unstable Töpfer's indicator colour in p_H 3·5 buffer, was used. For the Gunzberg titration, 5 c.c. aliquots were neutralized with 0·1 M NaOH, until no reddish tinge could be observed in any part of the test spot.

Acetone titration was conducted with 0·05 M NaOH on 2 c.c. aliquots to which about 18 c.c. of acetone and 4 drops of indicator had been added, end-points being matched against the colour controls I (p_H 3·4) and II (p_H 4). As an example, the data for the titration of mixture C, Table III, are presented :

Blank.—Equivalent salt concentration : 0·033 M (conditions at the end-point are referred back to the original aqueous solution). Titration of 2 c.c. 0·033 M NaCl in 18 c.c. acetone : to control II, 0·07 c.c. of 0·05 M HCl ; to control I, 0·23 c.c.

Mixture C.—Neutralization to control I, 1·14 and 1·13 c.c. of 0·05 M NaOH ; to control II, 1·30 and 1·31 c.c.

Corrected titres, I, 1·36 c.c., II, 1·37 c.c.

Known HCl titre of mixture C, 1·37 c.c.

% HCl neutralized, I, 99% ; II, 100%.

Formation of Precipitates.—The major technical difficulty of the titration is, of course, the insolubility of some of the constituents in 90% acetone. On titrating the originally acid solutions to the prescribed end-points no precipitation occurred, since NaCl, $NaNO_3$, and the acids examined were all soluble under the conditions. On the other hand, by continued neutralization precipitation occurred upon appreciable formation of the neutral salts, although acid salts were mostly soluble. Insolubility occurred most frequently with oxalates, sulphates, phosphates, and the amino acid zwitterions, the respective acid salts or hydrochlorides of these substances being satisfactorily soluble. Conversely, in back-titration of neutral salts, precipitation is avoided if HCl is added just short of the equivalent amount before adding acetone, and likewise, precipitation of protein and similar material is minimized by pre-acidification. Thus for all biological material it is advisable to acidify almost to the end-point before adding acetone for titration.

Results.

Titrimetric examples will be most valuable if they define the limits of availability of the method ; consequently the tables largely portray selected limiting cases. Table I has already demonstrated the extreme lack of specificity for free HCl of certain current methods. Sahli's method was shown to give partial estimation even of CO_2. On the other hand, the Gunzberg and thymol blue methods were more selective, but were technically difficult.

In Table II, data assisting the selection of indicators for the acetone titration are arranged. Equivalent amounts of oxalic, lactic, or acetic acids were mixed with HCl, and the percentage HCl neutralized for full colour change of the indicator observed. The data represent general magnitudes only. Naphthylamine orange is plainly the best indicator of those examined, the accurately determined end-point for HCl-oxalic acid being 97·5 to 101% HCl between the colour standards I and II, representing 100·5 to 102% titration if corrected for free HCl. For titrating weaker acids without the necessity of a free HCl correction, chrysoidine shows too poor a colour change, but neutral red or even bromphenol blue will serve for a sharp end-point with acids like acetic acid.

Table II.—Examination of indicators for acetone titration. Data as % HCl.

Indicator.	Type.	Aqueous range.	HCl-oxalic acid.	HCl-lactic acid.	HCl-acetic acid.
Töpfer's indicator	Basic	2·9–4·0	0	—	—
Methyl orange	,,	3·1–4·4	0	—	—
Methyl red (1st)	,,	4·2–6·3	0	—	—
Thymol blue	Acidic	1·2–2·8	70– 90	—	—
Congo red	Basic	3 –5	50–110	—	—
Benzene-azo-α-naphthylamine	,,	3·7–5·0	80–100	—	—
Erythrosin	Acidic	2 –3	90–110	—	—
Naphthylamine orange	Basic	4·2–5·8	92–102	94–100	94–100
Chrysoidine	,,	4·0–7·0	125–140	—	—
Neutral red	,,	6·8–8·0	185–193	102–110	100–101
Bromphenol blue	Acidic	3·0–4·6	190–195	110–135	100–101
Bromcresol green	,,	3·8–5·4	196	180–190	106–115
Cochineal	,,	4·8–6·2	197	187–195	110–115
Methyl red (2nd)	,,	4·2–6·3	198	193–197	—

In Table III, representative strong and moderately strong acids are examined, singly and in simple mixtures, by relevant methods. It is clear that acetone titration considerably improves the quantitative accuracy, and especially, the technical facility of the end-point. Thus even though end-points I and II have not the admirable definition of titrations in the region of neutrality, they may be fairly matched against the standard colours with an accuracy of ± 0·03 c.c. of 0·05 M acid or alkali. Substances for which major difficulty may be expected are nitric, sulphuric (its second ionization), oxalic, and the amino acids, whose $p_{K'}$ values estimated from the % titration are about 1·7, 3·3, 5·4, and 5 respectively in 90% acetone. Obviously, sulphuric acid is inimical to the titration unless more alkaline end-points may be used. Normally it must be removed if present in significant concentration. The other acids, however, are sufficiently excluded from partial titration, especially at end-point I, and cause no great trouble. Weak aromatic bases capable of interference are unlikely to be present. Thus, if suitable end-points are adopted, experimental error even under the most difficult circumstances is reduced effectively to under 5%.

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Table III.—Improved selectivity by acetone titration.

Solution.	p_{K_1}'.	Acetone Titration		Gunzberg reagent.	Thymol blue.	
		I p_H 3·4.	II p_H 4·0.		p_H 2·0	p_H 2·3.
Mixtures (data as % HCl)—						
0·05 M HCl, 0·025 M oxalic acid	—	101	102	—	—	-
0·05 M HCl, H_3PO_4	—	100	101	135	120–127	143–150
Mixture A	—	102	106	107	73–83	105–113
,, B	—	101	106	130	110–120	140–150
,, C	—	99	100	120	85–95	110–120
Acids (data as % one acid group)						
0·1 M HCl	—	100	100	99	76–81	85–89
0·1 M HNO_3	—	98	99·5	99	77–82	85–89
0·05 M H_2SO_4	(1·6)	159	182	185–190	133–140	153–160
0·05 M oxalic acid	1·2	1	4	85–90	53–63	70–80
0·1 M phosphoric acid	2·0	0	0·5	25–35	35–43	55–60
0·05 M glutamic acid HCl	2·2	3	10	16–18	10–17	30–40
0·1 M glycine HCl	2·4	2	8	7–11	7–14	30–35
0·1 M pyruvic acid	2·5	0	1	+	—	—
0·05 M citric acid	3·0	0	0	+	0	1–5
Polypeptides (mean)	3·5	—	—	—	—	—
0·1 M lactic acid	3·9	0	0	—	0	0

Mixture A = 0·025 M HCl, glycine HCl, and lactic acid.
,, B = 0·033 M HCl, 0·017 M glycine HCl, phosphoric and lactic acids, 0·008 M glutamic acid HCl.
C = 0·033 M HCl, 0·017 M phosphoric and lactic acids, 0·008 M citric and malic acids.

Summary.

Current methods of estimating "free HCl" in acid mixtures are examined, reasons for preferring the Gunzberg method or a p_H 2 titration being adduced.

These methods are superseded in accuracy and simplicity by a titration in 90% acetone solution to successive end-points at p_H 3·4 and 4·0 using *p*-benzenesulphonic acid-azo-α-naphthylamine (described as naphthylamine orange) as indicator. The methods are compared on acid solutions of known composition.

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Electrometric Titration of Amino-acids in Aqueous-alcoholic Solution.

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The conception that substances exist which carry both positive and negative charges in the same molecule was first advanced by Bredig (1894) and Küster (1897) in connection with betaine and methyl orange respectively. Küster designated such substances zwitterions; since, however, these particular compounds fail to exhibit the essential ionic property of migration in an electric field they should rather be called zwittermolecules.

Later Bredig (1899) extended the conception to the aliphatic amino-acids, and the theory was again taken up in an interesting paper by Adams (1916); the most convincing arguments in its favour were, however, supplied by Bjerrum (1923). In the meantime the results of many investigations had afforded additional support for the hypothesis; the more essential of these have been discussed by Richardson (p. 121) and may be summarized as follows.

(1) The zwitterion theory explains the positions occupied by the dissociation constants of the amino-acids in relation to those of the corresponding aliphatic acids, amines and esters (Adams, 1916; Bjerrum, 1923; Ebert, 1925; Emerson and Kirk, 1930); no such reasonable explanation is available on the older theory.

(2) Certain reactions of the amino-acids, *e.g.*, that with mustard oils (Marckwald *et al*, 1891) and that with aldehydes (Birch and Harris, 1930) can *only* be satisfactorily explained on the basis of the zwitterion theory.

(3) The temperature coefficients of the dissociation constants of the amino-acids indicate that the constants hitherto called basic and acidic correspond, in fact, to the carboxyl and amino groups respectively (Kolthoff, 1932).

(4) The extraordinary increase in the dielectric constant of water brought about by the addition of aliphatic amino-acids indicates that the latter possess a strong permanent dipole moment, whilst according to the older conception of their structure they should only possess a weak dipole moment (Hedestrand, 1928).

(5) The ultra-violet absorption of aqueous solutions of the amino-acids is easily to be explained on the basis of the zwitterion hypothesis (Ley and Arends, 1932).

The present investigation deals with a comparison of the titration curves of the amino-acids in water and in aqueous alcohol, the results of which afford a further fundamental piece of evidence in favour of the zwitterion theory. In connection with these experiments the methods of titration of amino-acids in water and alcohol will be discussed.

Nature of Ampholytes in the Light of Modern Acid-base Theory.

According to Brönsted (1923) acids and bases are substances which can yield or absorb protons respectively. In connection with this definition two points are fundamental. Firstly, the conceptions of acid and base are applicable not to molecules only but also to ions; in this sense, for example, we must regard the ions NH_4^+, $C_6H_5NH_3^+$, and $CH_3CH_2NH_3^+$ as acids, such acids being designated cationic or charged acids; bases, on the other hand, are substances such as OH^-, $C_6H_5NH_2$, $CH_3CH_2NH_2$, CH_3COO^-. Secondly, according to this conception the hydrogen ions alone are of fundamental significance; hydroxyl ions are of special significance only in that they absorb protons more readily than any other ion.

Ampholytes are substances which are able both to absorb and to yield protons. Thus the ions $HPO_4^{=}$ and $H_2PO_4^-$ are ampholytes whilst a compound such as $Zn(OH)_2$ is not, since in this compound only the OH^- ion and not the molecule as a whole is able to absorb protons. In its fully hydrogenated condition an ampholyte is a polybasic acid, which only exhibits peculiarities in the relationships of its electrical charges. Whilst the dissociation of ordinary dibasic acid H_2A is characterized by the stages HAH, HA^-, and $A^=$, that of an ampholyte is defined by the scheme HAH^+, HA, and A^-. If zwitterionic characteristics are ascribed to an ampholyte the intermediate stage HA takes the form $^+HA^-$. Following Brönsted's conception the following definition may be made: an ampholyte is a polybasic acid of which the fully hydrogenated form carries a positive charge, or is in other words a cationic acid. According to this new definition the term ampholyte is, in fact, confined for the most part to those organic substances which have hitherto been designated ampholytes.

The great simplification which Brönsted has introduced into the acid-base theory ought also to find expression in the nomenclature of dissociation constants. It is simpler to speak of only one kind of constants, namely, those of acids.

Every acid-base equilibrium is defined by the equation

$$\text{Acid} \rightleftharpoons \text{Base} + H^+.$$

Every so-called basic dissociation can thus be calculated as the dissociation of the corresponding acid. It is therefore more convenient to speak of first, second, etc., dissociation constants than of basic constants, constants of hydrolysis and so on. In this way the significance and the value of a dissociation constant become independent of the interpretation of a titration curve.

Effect of the Nature of the Solvent on Acid Dissociation.

In a further important contribution Brönsted (1928) has shown that a fundamental difference between cationic acids and ordinary acids will be revealed on passage from a solvent with a high dielectric constant such as water to one with a lower dielectric constant such as alcohol. The lowering of the dielectric constant will cause an increase in the strength of the interionic electrical forces and in ordinary acids this will result in a depression of the dissociation since the undissociated acid is electrically neutral

$$HA \rightleftharpoons H^+ + A^-.$$

In cationic acids, on the other hand, this effect will not be produced since the undissociated acid itself carries a positive charge

$$HA^+ \rightleftharpoons H^+ + A.$$

Let us now apply these considerations to ampholytes. With an ampholyte of the simplest character and one which does not form zwitterions, the dissociation relationships are represented by the following equations:

$$HAH^+ \rightleftharpoons HA + H^+$$
$$HA \rightleftharpoons H^+ + A^-.$$

The first dissociation follows the scheme of a cationic acid and the second that of an ordinary acid.

On the other hand, the relationships for the zwitterionic form are:

$$HAH^+ \rightleftharpoons {}^+HA^- + H^+$$
$${}^+HA^- \rightleftharpoons H^+ + A^-.$$

The zwitterion will indeed correspond in its behaviour neither with a neutral molecule nor with an ordinary ion. Its mutually opposed charges will neutralize each other to a small extent only. In alcoholic solution the first dissociation will be depressed but not so strongly as for an ordinary acid. The second dissociation will also be somewhat lower, but here the effect will be very much less than with an ordinary acid since the zwitterion cannot be regarded as electrically neutral. This latter dissociation may best be compared with the association of the ions of neutral salts which increases in solvents with a low dielectric constant.

Difficulty of Comparison of Acidities in different Solvents.

It is, however, difficult to say precisely what is to be understood by acidity in a non-aqueous solvent or mixture of solvents. The p_H of a given solution is defined as the difference between the activity of hydrogen ions in that solution and the activity of hydrogen ions in a standard solution. There are thus in principle two possible modes of definition: (1) the standard solution may be regarded as a common standard for all solvents and may thus be used as a basis for a universal scale of acidity; against this it may be urged that the difference between ionic activities in different solvents is not thermodynamically defined and can scarcely be estimated (Guggenheim, 1930); (2) an arbitrary new standard may be established for each solvent and mixture of solvents; this can only be done, however, by abandoning the thermodynamic connection, in the sense of activity, between acidities in different solvents. In the work described below I have refrained from anticipating any definition and have expressed the measured potentials in millivolts. When the term p_H occurs in the discussion of results obtained with aqueous-alcoholic mixtures it must be understood that it is used conventionally and has no thermodynamic significance.

It is not to be expected that acids of the same type, whether electrically neutral or cationic acids, will exhibit the same ratio between their dissociation constants in non-aqueous solvents as in water. Rather is it to be assumed that in addition to the electrical forces, whose significance has been indicated above, the individual chemical properties of the acids and their ions will come into play. In practice, however, it appears that the electrical effect predominates and that the differences in behaviour between individual acids of the same type are small in comparison with the differences between electrically neutral acids on the one hand and cationic acids on the other.

The measurements in the present investigation have almost all been made in a mixture of 90% alcohol and 10% water (by volume). The solvation effect, which, in a homogeneous solvent, need only be considered at high concentrations of electrolytes (Hückel, 1925) is of much greater significance in this mixture. The electrical field which surrounds an ion has an orientating influence on the molecules of the solvent and this influence will vary with the nature of the solvent. For instance, the orientating influence will be stronger on the molecules of water than on those of alcohol. In aqueous-alcoholic mixtures ions with a high capacity for solvation will tend to attract molecules of water rather than of alcohol, and the ionic activity will be determined by the dielectric constant of water rather than by that of the mixture as a whole. This consideration makes the profound influence of small additions of water on the potential of an alcoholic cell understandable (Millet, 1927). A half-cell of hydrogen chloride in 90% alcohol, for instance, shows a very small potential difference when compared with an aqueous hydrochloric acid solution of the same concentration; if the concentration of water in the first half-cell be further reduced, however, the potential difference rapidly increases. This effect will vary with different ions in relation to their capacity for solvation, and the phenomenon constitutes a further serious difficulty in the way of the establishment of constants for these mixtures. It is therefore impossible to estimate true "dissociation constants" by potential measurements with such mixtures, for, even if the p_H could be accurately determined, the activities of the anions and of the zwitterions can neither be estimated nor calculated. It is also impossible from these measurements to extrapolate the values of the dissociation constants in pure alcohol.

The "constants" which are given by the measurements are well adapted to the characterization of titration curves; they may therefore be designated "titration constants." Since the factor by which these values must be multiplied in order to give the real "thermodynamic" constant varies, for the reasons given above, from acid to acid, these "titration constants" must only be compared with one another with the greatest caution. It can, however, be shown experimentally that acids of the same type react in a similar way if the dielectric constant of the solvent is changed. Great differences can be observed clearly between electrically neutral acids and cationic acids, and between ampholytes of the ordinary form and ampholytes of the zwitterionic form. Owing to the predominance of the dielectric effect it is possible to determine to which type a given acid belongs, by comparing the titration curves of the acid (1) in aqueous alcohol and (2) in water. It is further possible to use these curves for volumetric analysis.

Michaelis and Mizutani (1925) carried out some experiments to determine the dissociation of acids in aqueous alcohol. As ampholytes they only investigated glycine and the aminobenzoic acids. Their own interpretation of their results differs from that offered below although they also consider that their experiments prove the validity of the zwitterion hypothesis.

Experimental.

The solvent in most of these experiments consisted of a mixture of 90% ethyl alcohol and 10% water by volume; the alcohol used was distilled from silver hydroxide in order to remove aldehydes, dried with anhydrous copper sulphate and again distilled. In later experiments ordinary absolute alcohol was used without further purification, since no difference could be observed in the results when the above processes were omitted. The hydrogen electrode used was in the bell form of Michaelis and was platinized every day. Two points seemed to be essential in order to obtain constant potentials in aqueous-alcoholic solution; firstly, the solution to be measured must be free of oxygen; secondly, the hydrogen gas must be saturated with the solvent before it enters the solution to avoid evaporation of the solvent, with consequent lowering of the temperature and alteration of the proportion of alcohol and water, and the concentration of the solute. Hydrogen taken from a cylinder was passed through a wash-bottle containing aqueous potassium hydroxide then over heated platinized asbestos and finally through a calcium chloride tower. After bubbling through a vessel containing the solvent in use for the experiment it was led into the titration vessel. The latter was closed except for a hole through which a stirrer was introduced. As a reference electrode an attempt was made to use an aqueous-alcoholic calomel electrode, but the potentials were not constant and the conductivity was not very good, so that a saturated aqueous calomel electrode was substituted. Michaelis and Bjerrum also used this reference electrode. The question of the effects which phase boundary and liquid junction exercise when an aqueous reference electrode is used will be dealt with below.

The potentials were measured by a Cambridge Instrument Company potentiometer. The temperature in all measurements was between 17° and 18° C.

Effect of Junction Potentials.

Some experiments were carried out in order to determine the phase boundary and liquid junction potentials between an aqueous and an aqueous-alcoholic half-cell. The aqueous-alcoholic half-cell consisted of 0·01 *N* acetic acid and 0·01 *N* sodium acetate in 90% alcohol. The other half-cell was a saturated aqueous calomel electrode.

The bridge solution consisted of two parts: (1) aqueous salt solution, (2) salt solution in 90% alcohol. The aqueous solution was connected with the calomel electrode and the alcoholic solution was connected with the alcoholic acetate solution. Both salt solutions were in contact inside a 3-way tap. Those salts were chosen of which the anionic and cationic mobilities were most similar in water and in alcohol, viz., potassium chloride, ammonium nitrate, and lithium acetate. K^+ has mobilities 74·8 in water and 24·6 in alcohol; for Cl^- the numbers are 76·3 and 21·4 respectively, for NH_4^+ 74·3 and 22 and for NO_3^- 71 and 26. The above numbers for alcohol were taken from a paper by Ulich (1927); no figures for acetate in alcohol could be found. The numbers for alcohol refer to pure alcohol; for 90% alcohol the values will be somewhat higher. These numbers also show that the differences between the transport numbers of the anions and of the cations are larger in alcohol than in water, and therefore the chance of destroying undesirable liquid junction potentials by interposing a concentrated salt solution is much less in alcohol than it is in water. It should also be pointed out that these salts are less soluble in alcohol than in water, and therefore other ions which may be present in the cell play a larger part in the formation of a liquid junction potential. Table I indicates that the values obtained with different bridges differ by not more than 4 mV.; it also shows that if the ratio of the concentrations is altered at the phase boundary the potential of the whole cell is not noticeably changed; this fact seems to show that phase boundary potentials do not play a large part in these experiments. If the concentration of other ions (especially of hydrogen ions) in the cell is low, then the liquid junction potential will probably not be above 5 mV. In any case the liquid junction potential does not change very much with the p_H. This fact can be proved by comparing the experimental titration curves with the theoretical curves. At very low p_H the liquid junction potential is certainly much higher. The assumption that phase boundary potentials do not exercise an effect in aqueous and alcoholic solutions of inorganic salts is also supported by the experiments of Wild (1923).

The potential of the calomel electrode was measured against aqueous 0·1 *N* hydrochloric acid and the p_H of the latter solution was taken as 1·08. The potentials of the alcoholic solutions were expressed as p_H, the term being recognized to have no thermodynamic significance for reasons indicated in the introductory part of this paper, and calculated from the e.m.f. measurements as if we were dealing with an aqueous solution without any further correction. The apparent titration constants of the various acids were calculated according to known formulæ and the activity coefficients of the different ions were not taken into account. The exact values for all amino-acids in aqueous solution are known from the work of Schmidt and his collaborators (for references see Myamoto and Schmidt, 1931) and therefore titrations in water were only made for comparison with the results of alcoholic titrations.

Table I.—0·01 *N* Acetic Acid and 0·01 *N* Sodium Acetate in 90% Alcohol. Temperature 18°.

Bridge-solution.		mV.
Aqueous part.	Alcoholic part.	
(1) Sat. KCl	—	632
(2) 5·0 *N* NHN_4O_3	0·64 *N* NH_4NO_3	634
(3) 1·6 *N* NH_4NO_3	0·64 *N* NH_4NO_3	633·5
(4) 0·8 *N* NH_4NO_3	0·64 *N* NH_4NO_3	633·5
(5) 5·0 *N* NH_4NO_3	0·32 *N* NH_4NO_3	635·5
(6) 0·8 *N* NH_4NO_3	0·32 *N* NH_4NO_3	635
(7) 5·3 *N* CH_3COOLi	0·62 *N* CH_3COOLi	634
(8) 2·7 *N* CH_3COOLi	0·62 *N* CH_3COOLi	634
(9) 0·53 *N* CH_3COOLi	0·62 *N* CH_3COOLi	634
(10) 5·3 *N* CH_3COOLi	0·31 *N* CH_3COOLi	636
(11) 0·53 *N* CH_3COOLi	0·31 *N* CH_3COOLi	636

All measurements were made in 0·01 *N* solution. The substances were of known purity. The titrations were made with 0·1 *N* hydrochloric acid, or sodium hydroxide in 90% alcohol. Glutamic acid and histidine are so sparingly soluble in 90% alcohol that it was necessary to titrate them in 80% alcohol. For the same reason tyrosine could only be titrated in very acid and very alkaline ranges.

In order to obtain points of comparison between ampholytes and ordinary acids and ampholytes and cationic acids titrations of the following substances were made: acetic acid, benzoic acid, salicylic acid, ammonia, ethylamine, and aniline. The following amino-acids were investigated: glycine, alanine, tyrosine, glutamic acid, arginine, lysine, histidine, proline, and hydroxyproline.

Table II.—Different Behaviour of Ordinary and Cationic Acids.
Temperature 18°.

	p_K Water.	p_K Alcohol.	Difference.	p_K Alcohol (Michaelis).
Acetic acid	4·68	6·92	+2·24	7·10
Benzoic acid	4·28	6·79	+2·51	7·03
Salicylic acid	3·00	5·10	+2·10	5·42
Ammonium (NH_4^+)	9·48	8·87	−0·61	8·87
$C_6H_5CH_2NH_3^+$	10·89	10·02	−0·87	—
$C_6H_5NH_3^+$	4·80	4·00	−0·80	4·02

Discussion of the Results.

Table II shows that the numerical values of the p_K of ordinary acids in alcohol are higher by 2·24 to 2·51 units than in water while the values of the p_K for cationic acids are lower by 0·61 to 0·87 units in alcohol than in water. This statement is valid not only for the acids mentioned here but also for many other acids which have been investigated by Michaelis and Mizutani (1925) and Mizutani (1925).

Fig. 1 shows an electrometric titration of ammonium acetate in alcohol and in water. The acid part represents a curve of an ordinary acid, acetic acid, *i.e.*, the alcohol curve is above the water curve. In the alkaline range the titration of the cationic acid NH_4^+ is demonstrated; here the alcohol curve is below the water curve.

It was deduced above that the first titration constant of ampholytes of non-zwitterionic form must have the same properties as the titration constant of an ordinary acid. That is to say, the numerical value of p_{K_1} must decrease on transition from water to alcohol, and that of p_{K_2} must increase. These deductions are substantiated for the aminobenzoic acids which according to Bjerrum do not possess zwitterionic constitution. In this paper, the titration curve of *o*-aminobenzoic acid only is presented, but the curves of the *m*- and *p*-isomerides are similar and are in accordance with the theory advanced. Fig. 2 shows clearly that in the acid range the alcohol curve is below the water curve, and is therefore characteristic of a cationic acid; in the alkaline range the alcohol curve is above the water curve, which is typical for an ordinary acid. Therefore p_{K_1} must correspond to the dissociation of the amino group, whilst p corresponds to the dissociation of the carboxyl group. It was

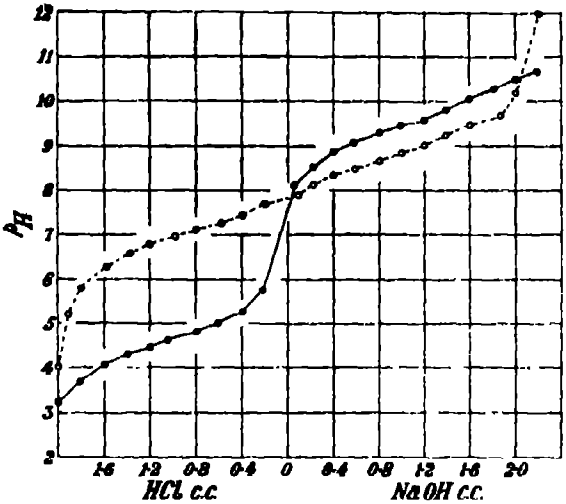

FIG. 1.—Ammonium acetate. ○ Alcohol; ● water.

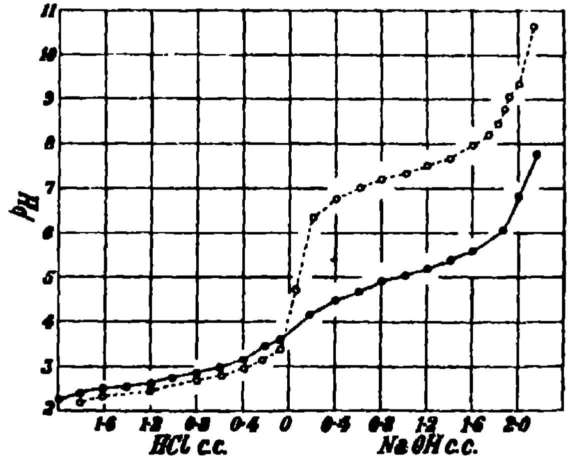

FIG. 2.—*o*-Aminobenzoic acid. ○ Alcohol; ● water.

further deduced above that an ampholyte of zwitterionic form must have p_{K_1} similar to the p_K of an ordinary acid and p_{K_2} resembling more that of a cationic acid. But for reasons which have been stated already, the increase of p_{K_1} which is obtained by lowering the dielectric constant of the medium will not be so great as for ordinary acids, and p_{K_2} will also be relatively a little greater than for cationic acids. As Table III shows glycine satisfies all these conditions. The p_{K_1} is higher in alcohol than in water, but the difference is smaller than with ordinary acids; p_{K_2} is nearly the same for both solvents, *i.e.*, the difference between p_{K_2} for alcohol and for water is relatively smaller than it is for cationic acids.

Table III.—Glycine and Aminobenzoic Acid.

	Temperature.	Water.	Alcohol.	Difference.
o-Aminobenzoic acid—	°			
p_{K_1}	18	2·12	1·92	−0·20
p_{K_2}	18	5·00	7·41	+2·41
Glycine—				
p_{K_1}	18	2·45	4·11	+1·66
p_{K_2}	18	9·88	9·99	+0·11
p_{K_1}	0	—	4·12	—
p_{K_2}	0	—	10·34	—
Glycine + 0·04 *N* LiCl—				
p_{K_1}	18	—	4·08	—
p_{K_2}	18	—	9·95	—

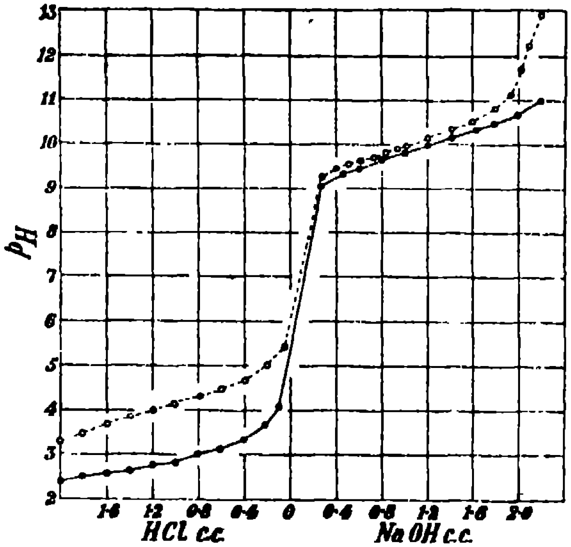

Fig. 3.—Glycine. ○ Alcohol; ● water.

Fig. 3 represents the titration of glycine, the acid part of the curve is similar to that of an ordinary acid, but the difference between the alcohol curve and the water curve is not so large as for ordinary acids like acetic acid, fig. 1. The alkaline range of the curve is more like that of a cationic acid. But whereas with a cationic acid such as NH_4^+, fig. 1, the alcohol curve is below the water curve, here both curves are close together. These facts are in accordance with the theoretical considerations in the introductory part of the paper. For glycine p_{K_1} corresponds to the dissociation of the carboxyl group, whilst p_{K_2} corresponds to the dissociation of the amino group.

In Table IV are shown the detailed results of the titration of glycine in alcohol, in order to illustrate the methods which have been used for the esti-

Table IV.—Titration of 0·01 *M* Glycine in 20 c.c. of 90% Alcohol. Temperature 18°.

	mv.	p_H.	p_{K_1}.
A. 0·1 *N* HCl—			
0·2 c.c. HCl	538	5·08	4·13
0·4 ,,	517	4·72	4·12
0·5 ,,	508	4·56	4·10
0·6 ,,	502	4·46	4·10
0·7 ,,	497	4·37	4·10
0·8 ,,	492	4·28	4·10
0·9 ,,	487	4·20	4·11
1·0 ,,	482·5	4·12	4·12
1·2 ,,	473	3·95	4·12
1·3 ,,	468	3·86	4·10
1·5 ,,	458	3·69	4·11
1·7 ,,	448	3·52	4·16
1·8 ,,	443	3·43	4·15
1·9 ,,	438	3·34	4·12
2·0 ,,	433	3·25	—
2·1 ,,	427	3·15	—
2·2 ,,	420	3·03	—
B. 0·09 *N* NaOH—			
0·3 c.c. NaOH	774	9·10	9·98
0·4 ,,	786	9·40	10·01
0·5 ,,	793	9·52	10·00
0·6 ,,	799	9·62	9·99
0·7 ,,	805	9·72	9·98
0·8 ,,	810	9·79	9·97
0·9 ,,	815·5	9·90	10·00
1·0 ,,	820	9·99	10·00
1·1 ,,	825	10·07	9·99
1·2 ,,	830	10·17	10·00
1·4 ,,	840	10·35	10·00
1·5 ,,	848	10·47	10·01
1·6 ,,	854	10·58	10·00
1·7 ,,	861	10·71	9·99
1·8 ,,	872	10·90	9·99
1·9 ,,	885	11·12	(9·88)
2·0 ,,	910	11·60	—
2·1 ,,	950	12·25	—
2·2 ,,	991	12·96	—
2·3 ,,	1010	13·29	—

mation of titration constants in this paper. It must be pointed out that there exists in solution an equilibrium between zwitterionic molecules and non-zwitterionic molecules. The concentration of the non-zwitterionic form in water is very small; according to the calculations of Myamoto and Schmidt (1932) the ratio is probably about 1 to 10^5. In alcohol the concentration of the non-zwitterionic form is certainly higher (see also Ebert, 1925), but nevertheless the zwitterionic form also predominates here, as can be shown by determining the temperature coefficient of the two p_K. The p_K of a carboxyl group does not change noticeably with the temperature while the p_K of an amino group changes considerably. Table III shows that the values of p_{K_1} measured at 0° and at 18° do not differ; the difference between the two values of p_{K_2} is large and indicates that in alcohol the zwitterionic form also predominates.

Another point must be taken into account: since amino-acids cause the dielectric constant of water to be increased it is possible that this effect also takes place in the case of aqueous alcohol. Hedestrand (1928) found that the addition of neutral salts to an aqueous amino-acid solution reverses this effect and lowers the dielectric constant of the solution even below the value of the pure solvent; the influence of the addition of lithium chloride on the p_K of glycine was therefore investigated. But Table III shows that the differences are within the limits of experimental error and may be due to the alteration of the ionic strength of the solution.

Table V indicates that p_{K_1} of all amino-acids, which according to the zwitterion theory corresponds to the carboxyl group, shows the same behaviour as p_{K_1} of glycine. The values in alcohol are higher than in water, the differences vary generally between 1·60 and 1·79 units. It is peculiar that with histidine and tyrosine the differences are small; figs. 4 and 8 illustrate this. It can be seen that in alcohol the conditions for the titration of the carboxyl group are much more favourable than in water, because the blank-correction is not so large. The dissociations of all α-amino-groups, p_{K_2} of alanine, tyrosine, lysine, arginine and p_{K_3} of glutamic acid and histidine resemble that of the amino group of glycine. Alanine has about the same values as glycine. It has been already mentioned that tyrosine, fig. 4, has a relatively low p_{K_1} in alcohol, possibly because the zwitter-form is practically insoluble in 90% alcohol, and this is also the reason why only a part of the curve in alcohol can be presented.

But the difference between the basic amino group and the acid phenolic group can be clearly shown. Whilst in water the values and the curves are very close to each other, in alcohol, even if it is not possible to calculate p_{K_2}, fig. 4 shows that the curves are quite distinct; this proves the assumption that p_{K_2} corresponds to the amino group and p_{K_3} to the phenolic group. Fig. 5 represents the titration of a dicarboxylic acid. The differences in the p_K values of both carboxyl groups in alcohol and water are about the same. In lysine, fig. 6, both amino groups show the same basic characteristics. The

Table V.—Temperature 18°.

	Water.	Alcohol.	Difference.
Alanine—			
p_{K_1}	2·48	4·27	+1·79
p_{K_2}	9·96	10·09	+0·13
Tyrosine—			
p_{K_1}	2·35	3·38	+1·03
p_{K_2}	9·16	—	—
p_{K_3}	10·41	12·45	+2·04
Glutamic acid—			
p_{K_1}	2·15	3·80	+1·65
p_{K_2}	4·28	5·70	+1·42
p_{K_3}	9·70	9·85	+0·15
Lysine—			
p_{K_1}	2·20	3·93	+1·73
p_{K_2}	9·18	9·36	+0·18
p_{K_3}	10·78	10·92	+0·14
Arginine—			
p_{K_1}	1·76	2·88	+1·12
p_{K_2}	9·25	9·24	−0·01
p_{K_3}	12·75	13·00	+0·25
Histidine—			
p_{K_1}	1·76	2·88	+1·12
p_{K_2}	5·99	6·10	+0·11
p_{K_3}	9·30	9·28	−0·02
Proline—			
p_{K_1}	2·00	3·70	+1·70
p_{K_2}	10·62	10·55	−0·07
Hydroxyproline—			
p_{K_1}	1·95	3·55	+1·60
p_{K_2}	10·00	10·10	+0·10

titration constant of the guanidino group of arginine, fig. 7, as a basic group has similar numerical values for alcohol and water; but owing to the fact that the whole p_H-range is enlarged in alcohol the blank correction is much less and the curve is more clearly defined. In histidine, fig. 8, the p_K of the glyoxaline group shows only a slight difference in alcohol and water in accordance with its basic character.

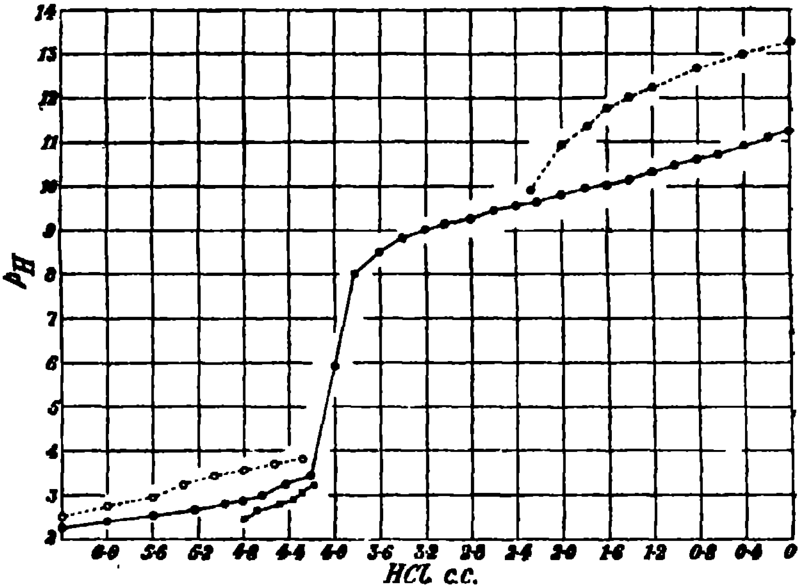

Fig. 4.—Tyrosine, disodium salt. ○ Alcohol; ● water; ■ water correction.

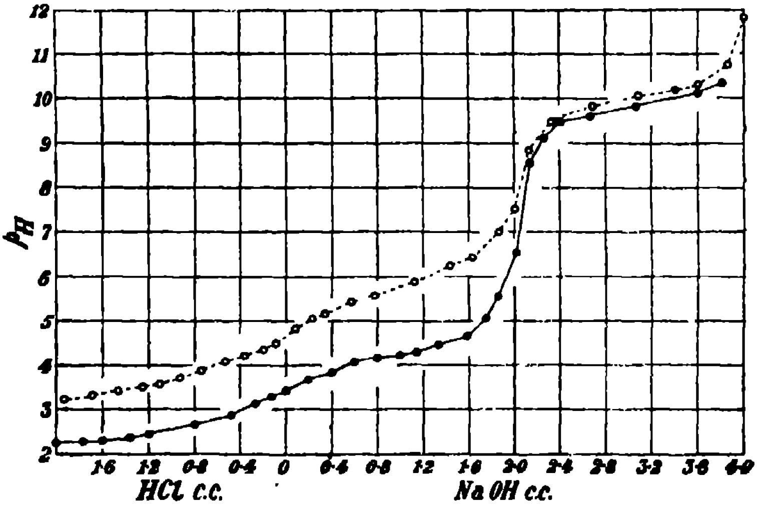

Fig. 5.—Glutamic acid. ○ Alcohol; ● water.

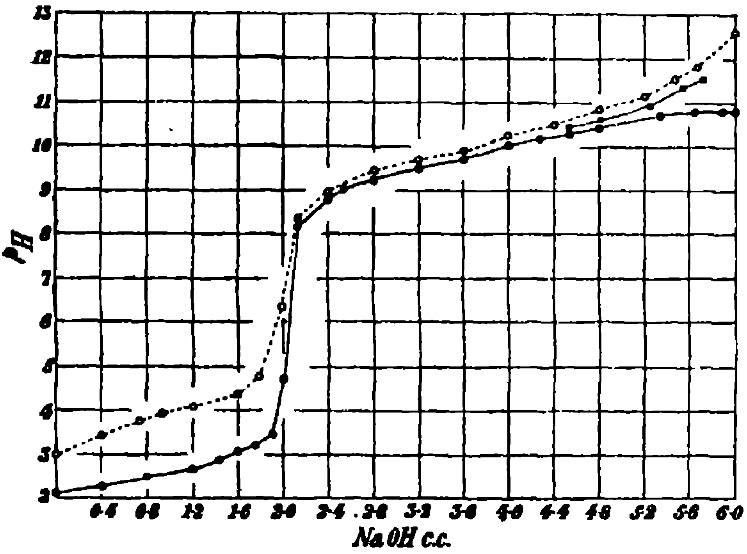

Fig. 6.—Lysine dihydrochloride. ○ Alcohol ; ● water ; ■ water correction.

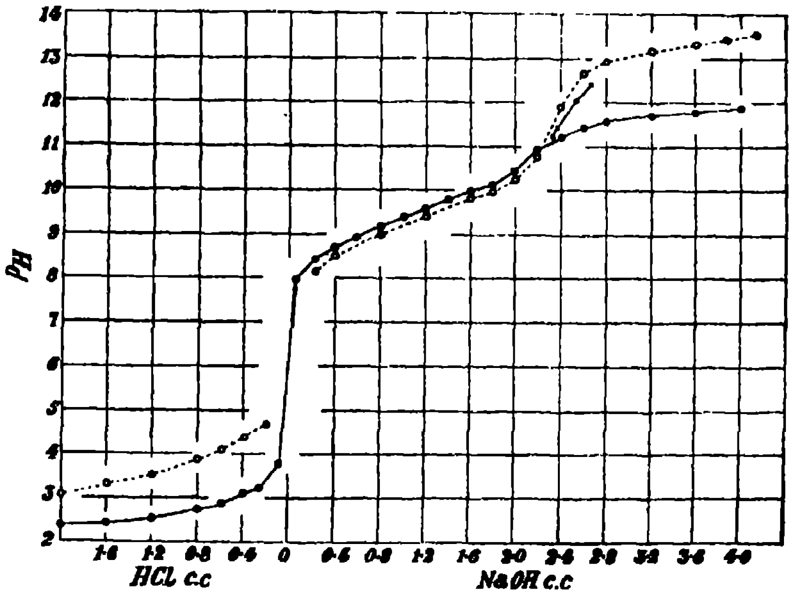

Fig. 7.—Arginine monohydrochloride. ○ Alcohol ; ● water ; water correction.

From the values for proline, fig. 9, and hydroxyproline it can be shown that imino-acids are also zwitterions. The values of p_{K_1} in alcohol and water for both these acids show that they correspond to the carboxyl group whilst p_{K_2} is the constant of the imino group.

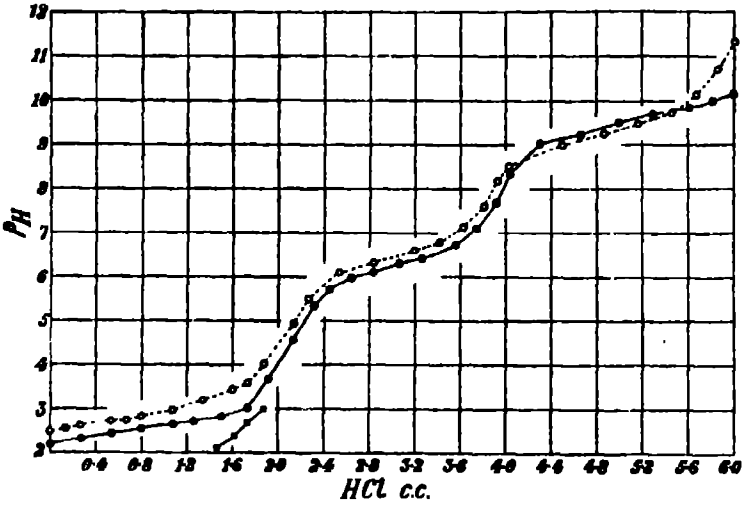

FIG. 8.—Histidine dihydrochloride. Alcohol ; ● water ; ■ water correction.

General Discussion.

It seems necessary to emphasize the general importance of titrations in aqueous alcohol and the comparison of these alcoholic curves with the corresponding curves for aqueous solutions.

First of all the whole p_H range becomes larger with decreasing dielectric constant and therefore very weak acids which are difficult to titrate in water in a satisfactory manner are easily titrated in aqueous alcohol. Examples are the titrations of the α-amino group, figs. 3 to 8, the imino group, fig. 9, the ε-amino group, fig. 6, and to some extent the guanidino group, fig. 7. Secondly, the different behaviours of basic and acid groups which are found when the dielectric constant of the solution is changed enables us to separate groups whose dissociation constants are very close together and this point has some importance for the interpretation of dissociation constants (see Richardson 1933) and for analytical purposes. An example in this paper is tyrosine fig. 4.

The application of the zwitterion theory to the methods of determining amino groups or carboxyl groups in proteolytic studies has been made by

Richardson (1934). The alcoholic titration of Foreman (1920) and Willstätter and Waldschmidt-Leitz (1921) is an estimation of the α-amino group. The discussion as to whether the addition of alcohol causes an increase of the acidity of the solution has no real significance because the "acidity" of an aqueous-alcoholic solution is an arbitrary magnitude.

In comparison with an ordinary acid the dissociations of all cationic acids and basic groups are higher in alcohol; only in this sense is it possible to speak of a stronger acidity in alcohol.

The advantages of the application of alcohol in estimating amino groups are twofold. Firstly, whereas in water the p_K of the amino groups is so high that it is difficult to titrate them, in alcohol the conditions are more favourable; the numerical value of the p_K remains about the same as in water on account of the zwitterionic character of the amino-acids and the p_H range becomes much larger which makes the titration more satisfactory. Secondly, other groups, *e.g.*, the phenolic group, which disturb the titration in water cannot have that effect in alcohol. The Linderstrøm-Lang (1928)* titration is an estimation of the carboxyl groups and is also connected with the zwitterionic character of the amino-acids. The carboxyl groups are very acid in water and the acidity decreases with the lowering of the dielectric constant of the medium. If the

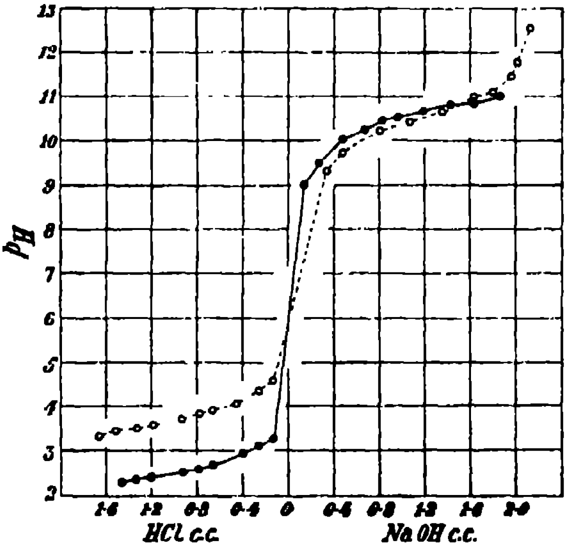

Fig. 9.—Proline. O alcohol; ● water.

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* Linderstrøm-Lang in his paper also gives a theoretical explanation which takes the dielectric constant into account.

amino-acids had not a zwitterionic character the conditions would be as unfavourable as they are in water.

It is my intention to apply these electrometric titration methods to proteolytic investigations.

I wish to express my gratitude to Professor C. R. Harington, F.R.S., for his continued interest and to thank the Academic Assistance Council for a personal grant.

Summary.

(1) It has been shown that the acid-base theory of Brönsted can be made the basis of a simple theoretical treatment of the constitution of ampholytes.

(2) A technique has been developed by which aqueous-alcoholic solutions of amino-acids may be titrated electrometrically against a standard aqueous calomel electrode with very little interference from liquid junction or phase boundary potentials.

(3) Although the system used cannot be thermodynamically defined it is capable of showing clearly the variation in the behaviour of acidic and basic groups in relation to the dielectric constant of the solvent.

(4) The results obtained by this method give strong support to the zwitterionic theory of the constitution of amino-acids.

(5) The importance of electrometric titrations in aqueous alcohol for volumetric purposes and for the interpretation of dissociation constants has been pointed out and the basis of the existing volumetric methods of estimation of amino-acids has been discussed in the light of the facts revealed by the titration curves.

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Nerve Heat Production as a Physiological Response to Excitation.

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For many years attempts were made to record the energetic and metabolic changes in nerve associated with activity. Since 1926, when the heat production was first measured, a considerable amount of work has been published on the chemical and thermal changes and on the oxygen consumption and carbon dioxide production caused in nerve by stimulation. The regularity of the results obtained under all kinds of conditions (*e.g.*, of frequency and duration of stimulus, of temperature, of the presence and absence of oxygen) and the general agreement between heat and oxygen measurements on the one hand and the electric response on the other, seemed to give assurance that a genuine physiological characteristic was being investigated. It has, however, been persistently objected by H. Winterstein that the increase observed, as the result of electric excitation, in the metabolism of nerve is simply a direct consequence of the electric current passed through it and has nothing whatever to do with the normal process by which the impulse is propagated. This drastic view has been embodied in a text-book article (1932) in which "the regrettable conclusion" is reached that "the wonderful technique" developed for studying the heat production of the nervous system has led to no results of value as to the energetics of the physiological process of excitation.

The experiments on which this opinion is based have been criticized in detail by Gerard (1932, where full references will be found), by Meyerhof and Schulz (1930), by Schmitt (1933), by Hill, Fenn and Gerard (1934), by Harashima (1933), by Gerard and Hartline (1934), and by Hill (1932, *a*, *b*). It can easily be calculated, moreover, that in the later experiments by Winterstein and his colleagues (*e.g.*, Ledebur, 1933) on "reflex excitation" the method employed was so unreliable and the quantities to be observed so small that the negative results found were to be expected in any case. Since, however, the "regrettable conclusion" might, in spite of the evidence, confuse the issue the experiments to be described have been made in the hope of deciding the matter.

The heat production of stimulated nerve, in all the latest experiments (Hill, 1933; Bugnard, 1934; Beresina and Feng, 1933), was measured in a region of nerve the nearest point of which was 35 to 40 mm. below the stimulating electrodes. The stimuli were condenser discharges of known time characteristics, approximately "optimal," and they were always somewhat submaximal. Between the stimulating electrodes and the thermopile was a large silver block on which the nerve lay in a groove, and in many of the experiments this block was earthed. The electrodes were about 2½ mm. apart (see fig. 1). Under the circumstances it was very improbable that the stimulating current would spread 40 to 65 mm. to the nerve lying on the thermopile, and with such strength and regularity as to produce, simply as an artefact, the very consistent results recorded.

It was not difficult, however, to test the possibility by stimulating (*a*) at electrodes closer to the thermopile, and (*b*) at more distant electrodes, and comparing the results. When the silver plate (fig. 1) alone was earthed there was no chance of the stimulating current spreading beyond it to go to earth; it could go beyond it only in the outer fringe of a distribution of closed current lines around and between the electrodes. The density of this outer fringe of current must fall off extremely rapidly at greater distances from the electrodes—as does the magnetic field round a small magnet, which at points along the axis varies inversely as the cube of the distance. Hence, by using a pair of electrodes closer to the thermopile the current-spread to the nerve in the region investigated would be greatly increased, and a greater thermal response obtained. If reducing the distance had no effect on the heat production it would be difficult to attribute the latter to current-spread, or indeed to any *direct* (as distinguished from *transmitted*) effect of the stimulus.

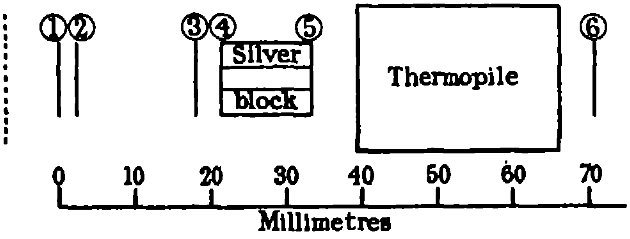

FIG. 1.

A thermopile was prepared as in fig. 1, with a pair of adjustable electrodes (1) and (2) generally used at a distance of 35 to 40 mm. from the nearer end of the thermopile, and a third electrode (3), which, with the edge (4) of the silver block, could be used as a second pair at about half the distance from the nearer end of the section of nerve in which the heat was measured. The nerves stretched about 7 mm. above (1) (2), so that stimulation occurred well away from the cut end. Their lower ends reached just beyond the fixed electrode (6) which, with the edge (5) of the block, was used only for calibration and otherwise was disconnected.

In no experiment was any significant difference found, applying the same stimulus (*a*) at (1) and (2), and (*b*) at (3) and (4). With alternating shocks and with one-way shocks the results were identical. It might be argued that the shocks applied were enormously supermaximal for most of the fibres and so could produce a maximal artefact even at a considerable distance. This was tested by finding the relation between response and strength of stimulus (*a*) at electrodes (1) and (2), and (*b*) at electrodes (3) and (4). The results are given in Table I. The stimulus was altered by changing either its voltage (January 3, January 1) or its capacity (January 8). It is evident that the relation between stimulus and response was practically the same whichever pair of electrodes was used; so far as there was a difference at all, the greater response was obtained from the more distant electrodes.

When a response is about half maximal, its size is very sensitive to small changes in the stimulus. In spite of this, Table I shows that from a given stimulus practically the same response was obtained from both pairs of electrodes. With the sub-maximal stimuli, were the response observed due to any direct effect of the stimulating current it could not fail to be greater from the nearer electrodes. The results, however, are simply and naturally explained if we regard the observed response as due to a propagated disturbance.

The observed heat, therefore, is due to a propagated and not to a direct effect of the stimulus. It remained, however, to establish the heat production as a normal physiological factor, namely, to show parallelism under certain critical circumstances to other known characteristics of nerve response. The power of a propagated impulse to initiate muscular contraction is universally admitted as a normal physiological function; and it is too late to-day to deny the existence of the action potential accompanying the propagated disturbance as a normal effect of a "natural" stimulus. It was required, therefore, to find some characteristic of the heat production of nerve which would allow it to be compared (*a*) with the mechanical response and (*b*) with the action current. With the latter, as a matter of fact, it has already been directly compared by Gerard, Hill and Zotterman (1927): as the frequency of excitation was altered the heat production in a short stimulus varied in the same way as the total electrical response.

Table I.—Relation between strength of stimulus and thermal response of nerve at near and far electrodes.

Experiment of January 3, 1934.—Hungarian frogs' nerves, 20·4° C. Nerve resistance between electrodes (1) and (2) 1900 ohms, between electrodes (3) and (4) 2700 ohms (nerves thinner at (3) (4) than at (1) (2)); measured by bridge, employing condenser discharges as used for stimulation, and a telephone. Stimuli, 16 seconds' duration, 0·506 μF, shunt of 200 ohms across electrodes, no series resistance. Shocks about "optimal," since RF = about 100.

(A) *Alternating shocks* 885/*sec.*

Volts	0·61	0·82	1·03	1·23	1·64	2·05	2·44	2·82	3·05	4·10	2·82	2·05	1·64	1·23
Response. stimulation (1) (2)	2	6	10	17½	23½	27	28	29½	35	36	29	26½	24½	17
Response: stimulation (3) (4)	0	4½	8½	11½	19	24	26½	29½	29½	29	27	23	19	14

(B) *One-way shocks, 245/sec., cathode at* (1) *or* (3).

Volts	0·61	0·82	1·03	1·23	1·64	2·05	2·45	3·28
Response: stimulation (1) (2)	2	9	14½	17½	24½	24½	27	29
Response: stimulation (3) (4)	5	10½	13	18½	23½	26½	26	29

(C) *At this frequency, stimulating at electrodes* (1) *and* (2), *the cathode was taken alternately at* (1) *and* (2).

Volts	2·45			1·23	
Response: cathode (1)	26	25	25	15	14½
Response: cathode (2)	29	28		17½	17

(D) *One-way shocks, 440/sec., cathode at* (1) *or* (3).

Volts	2·05	1·03
Response: stimulation (1) (2)	25½	9½
Response: stimulation (3) (4)	23½	11½

(E) *Continuous stimulation*, 0·815 μF, otherwise as above, 53 one-way shocks per second, switched alternately from electrodes (1) (2) to electrodes (3) (4) and back. Cathode (1) or (3). Threshold stimulus about 0·6 v. With 0·8 v. for 16 minutes the results were indistinguishable, both about 40 mm. With 1·03 v. for 8 minutes the deflection reached 70 mm., and again the results were indistinguishable. With 1·24 v. for 8 minutes the deflection reached 82 mm., and cathode (3) gave slightly the greater effect.

Experiment of January 1, 1934.—Hungarian frogs' nerves, 20° C. Nerve resistance between electrodes (1) and (2) 1800 ohms; between electrodes (3) and (4) 2500 ohms. Stimuli, 16 seconds duration, 0·196 μF, 1000 ohms shunt, 10,000 ohms series, shocks about "optimal" since RF = about 180. Alternating shocks, 895/sec.

Volts	2	4	6	8	10	12	14	18	18	14	12	10	8	6	4	2
Response: stimulation (1) (2)	0	11	21	27½	30	33½	35½	39	38½	34	32	30½	27½	21½	13	0
Response: stimulation (3) (4)	0	12	21	26	29	—	31	33	—	30½	—	28	25½	19½	12	0

Experiment of January 8, 1934.—Hungarian frogs' nerves at 27·50° C. Stimuli, 16 seconds' duration, one-way, 685/sec., shunt of 200 ohms across electrodes, no series resistance, 4 volts, various capacities. Cathode at (1) or (3).

Microfarads	0·0318	0·055	0·105	0·208	0·313	0·414
Response: stimulation (1) (2)	4	16	29½	35	40	41
Response: stimulation (3) (4)	6	15½	26½	33	38	38½

In this experiment, during 20 minutes' continuous stimulation with alternating shocks at 143 shocks/sec. (4 v., 0·313 μF., 200 ohms shunt) at electrodes (1) and (2), the stimulus was transferred four times for 1 or 2 minutes to electrodes (3) and (4) by turning over a key. There was no change at all in the deflection.

A direct comparison, however, between nerve heat and muscular response was not possible owing to two circumstances: (*a*) the nerve heat is so small that a prolonged stimulus must be given and the muscle responding to this stimulus would then be soon fatigued; (*b*) the muscle itself liberates so much heat that if it were in the same chamber attached to the nerve the heat from it would entirely mask that due to the nerve itself. A characteristic therefore was chosen which enabled an indirect but very pertinent comparison to be made between the three different effects, namely, the time factor in excitation. If we could find the relation, for a given response of any kind, between the strength of a stimulating shock and its duration, and if this relation proved to be the same for two or more different forms of response, we could naturally assume that these were intimately connected. To be specific, if the strength-duration curve for nerve excitation were found to be the same when the response looked for was heat, as when the response looked for was a muscular contraction, it would be very difficult to deny that the heat was directly associated with the process by which the impulse was transmitted which caused the muscle to contract.

In fig. 2 (upper) is shown a series of eight curves relating the heat response of nerve in a 20-second stimulus to the time of discharge of the condenser used for stimulation. The experiment was made at 25° C. on Hungarian frogs acclimatized to that temperature. Each observed point as plotted represents the sum of the two responses in a series starting at the lowest voltage and working up to the highest and back again. No point has been omitted. One-way shocks were applied between electrodes (1) and (2), the cathode being at (2). The frequency 303/sec. was constant throughout. The electrodes had a 600-ohm shunt, which, with the series resistance and the measured resistance of the nerves, gave a total discharge resistance for the condenser of 508 ohms. This resistance was constant throughout and the time of discharge was varied only by varying the capacity of the condenser.

Each curve represents a different voltage. The right-hand curve was for 1·40 volts and the response was observed for six different capacities in increasing size. The voltage was then changed to 1·82 and the response measured with seven different capacities. The voltage was then altered to 2·39 and the response to eight different capacities found. The process was continued with 3·24 volts, 4·28 volts, 5·92 volts, 8·58 volts and 12·26 volts, the same being then repeated in the reverse order. The means were plotted, the response being regarded as a function of the logarithm of the time of discharge of the condenser. If F microfarads be discharged through R ohms then the charge has fallen to $1/e$ of its initial value in a time RF millionths of a second. For simplicity we will call the time required to discharge to $1/e$ of the initial value the "discharge time"; when $\log \mathrm{RF} = 3$ the "discharge time" is 1σ.

The advantage of logarithmic plotting is two-fold: (1) it enables all the curves to be drawn on the same diagram and to appear not only of about the same size but of about the same shape; and (2), as Rushton has insisted (1931), the most satisfactory scale for plotting strength-duration curves is logarithmic along both axes. It is a convenience in this form of plotting to have log RF already given.

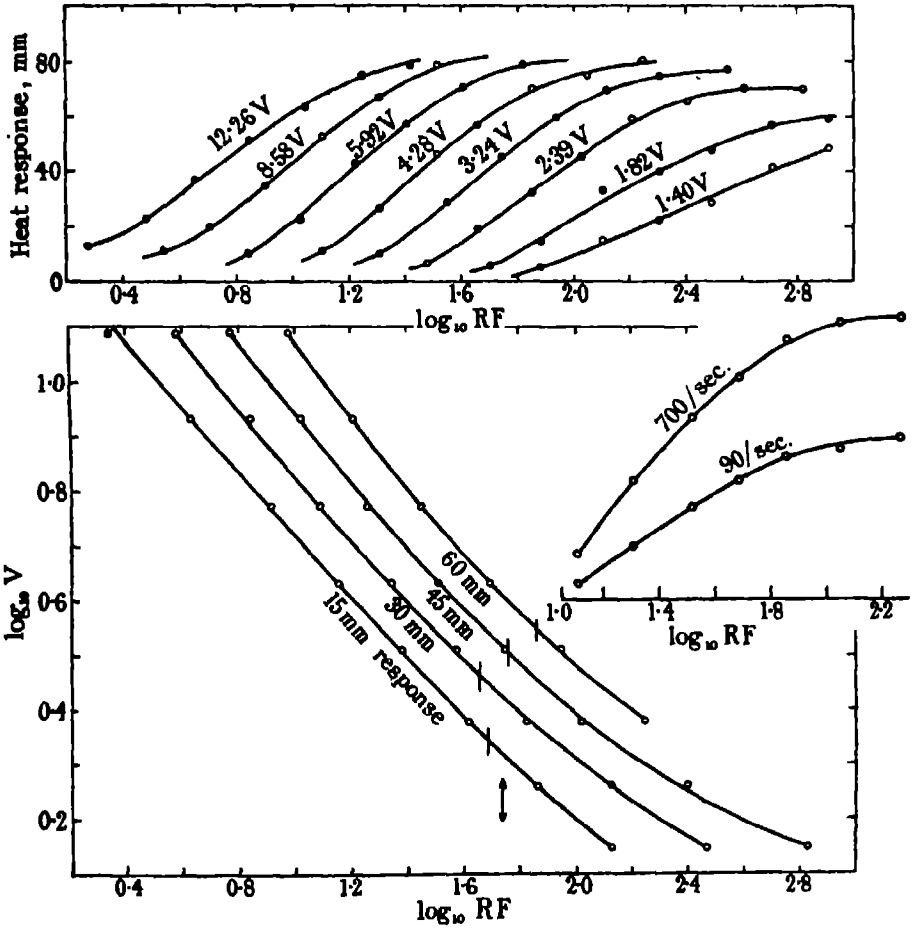

FIG. 2.

In the upper curves of fig. 2 we have all the information required to plot the "strength-duration curve" for any size of response. As usually defined, the strength-duration curve is recorded for a minimal muscle twitch: there is no virtue, however, in a minimal response—any constant response is as good. From the upper half of fig. 2 we may read off, for any given size of thermal response, the relation between the voltage and the discharge time, and this has been done in the lower half of the figure for four different heights of response. The lowest curve relates the logarithm of the voltage to log RF for a response of 15 mm. The eight circles are derived from the eight curves in the upper half of the figure. For a greater response a greater capacity is required for a given voltage, a greater voltage for a given capacity. Consequently the strength-duration curve for 30 mm. response lies above that for 15 mm. response. Similarly the curve for 45 mm. response lies higher again, and that for 60 mm. still higher.

It will be noticed that log V is plotted on twice the scale of log RF. It is a valuable characteristic of these curves, as Rushton has pointed out (1931, p. 286), that the "optimal" stimulus, *i.e.*, the stimulus of minimum energy, lies at the point where, with this scale of plotting, the curve has a slope of 45 degrees. In order, therefore, to obtain the optimal stimulus from these curves, all that is necessary is to place a parallel ruler on the diagram at 45 degrees to the axes, to move it forward and to mark the point at which it touches the curve. Such points are marked in fig. 2 by the four vertical lines crossing the curve and the arrow represents the mean of the four. The optimal stimulus here is such that log RF = 1·74, the half-discharge time of the condenser therefore being 0·038 σ.

It might have been suspected that the frequency would influence the shape and not only the size of the response-capacity curve given in the upper part of fig. 2. This is not noticeably so. In the small figure inset, two response-capacity curves are shown for two very different frequencies, namely, 90/sec. and 700/sec. respectively. For each the response is plotted as a function of log RF. The two curves are of the same shape but, of course, of different heights, the higher frequency giving the greater response.

It is not possible with nerve heat to continue the strength-duration curve to the extreme right. In order to obtain a sufficient response in a short stimulus it is necessary to employ rather a high frequency, and with one-way shocks from a commutator not more than about 40% of each interval is available for the discharge. This, therefore, has to be rather rapid or else it will not be complete in the interval of contact. If a lower frequency could be employed the strength-duration curve could be taken to considerably longer times. This, however, would not affect the determination of the optimal stimulus which, as Waller pointed out many years ago (1899) and as Lucas emphasized (1906, *a*, 1906, *b*), is an indicator of the time factor in excitation. (See also Cybulski and Zanietowski (1894)). The " excitation time " (or the " chronaxie ") is another quantitative expression of this same factor, but it has no advantage in principle over the duration of the optimal stimulus, so that the optimal stimulus, being more easily determined in the present case, can be taken as the indicator we require. It is simplest to express it by its logarithm, namely, as log RF for the stimulus of minimum energy. This is read off directly with the aid of a parallel ruler from such curves as those in the lower part of fig. 2.

There is nothing in the theory of excitation to suggest that the energy as such plays any specific rôle. Its choice is just as arbitrary as that of the factor 2 in the definition of the chronaxie. The quantity FV^n has a minimum for any value of n less than (about) unity, and the value of RF for which FV^n is a minimum can be used as a measure of the time factor in excitation. The " optimal " stimulus is that for which $n = 2$. It might in fact be better to adopt another value, *e.g.*, $n = 3$, and to find RF, for FV^3 to be a minimum. This could be read off on the log V - log RF curve from the point at which $d \log V/d \log RF = -1/3$; or, since the scale of log V in the figures is twice that of log RF, from the point at which the negative slope is 2/3. The point would be more to the right than for the " optimal " stimulus, and so on a rounder part of the curve where it could be determined with a parallel ruler with greater accuracy.

Several other experiments of this kind have been made. One of these is shown in detail in fig. 3. Hungarian frogs were acclimatized to 37° C. by keeping them for several days at a high temperature. The heat production was measured by the maximum deflection in a 16-second stimulus. The frequency was 350 one-way shocks per second at electrode (2). The total resistance of the discharge circuit of the condenser was 292 ohms, being made up of a 405 ohms shunt across nerves with a measured resistance of 1050 ohms. Various voltages were taken from 0·82 to 12·2, and for each voltage the response was found for a series of different capacities. As before, in the upper half of the figure, the response is plotted as a function of log RF. On the diagram a horizontal line is ruled at a height of 25 mm. At the points where this line crosses the several curves, volts and log RF can be read off for the given response. Log V is plotted against log RF in the right-hand bottom part of the figure (hollow circles). Here the optimal stimulus is for log RF = 1·76.

In the same diagram is given another curve (solid circles) for an experiment made under exactly similar conditions at 32°. The total resistance of the discharge circuit was 175 ohms, 16-second stimuli were provided with a frequency of 550 one-way shocks per second, and the plotted curve gives an optimal stimulus at the arrow with a log RF = 1·84.

There is another way in which the optimal stimulus can be determined from the data, namely, by reading off from each curve in the upper part of the figure the response for which FV^2 has a certain constant value. FV^2 is proportional to the energy of the stimulus, and if FV^2 be given any particular value, *e.g.*, 1·24, then for each value of V the value of F can be calculated and so the point determined for which the response is required. This method has been employed for a number of different values of FV^2 to construct the diagram in the lower left-hand side of fig. 3. The curves there given are exactly similar to those in a previous paper (Hill, 1932, *b*, p. 125) and the optimal stimulus obtained from them must, if the calculation and plotting be properly carried out, be the same as that found with the parallel ruler on the other type of

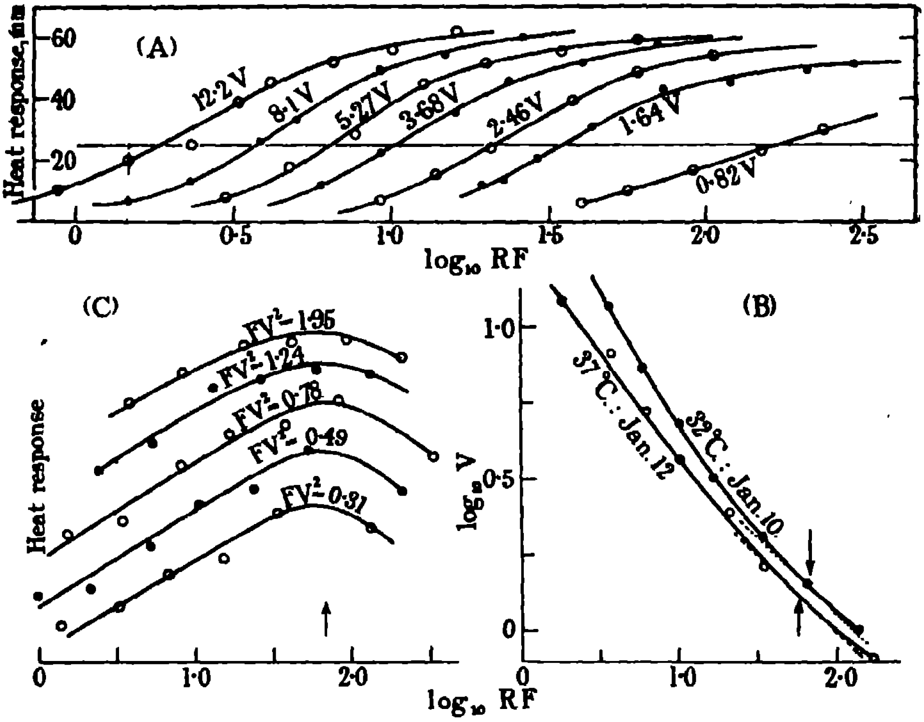

Fig. 3.

curve. Fig. 4 shows a much better example of the same curves, derived in this case from the experiment shown in fig. 2. The optimal stimulus is not so accurately determined from these curves as from those of fig. 2, but the value shown by the arrow corresponds to log RF = 1·80 which is not far from the mean value 1·74 found from fig. 2.

The same procedure can be carried out for the electrical response. With the assistance of Mr. Donald Scott the experiments were made which are shown in figs. 5, 6 and 7. A nerve was mounted in a paraffin wax chamber and a monophasic response recorded with the aid of calomel electrodes and a Downing moving magnet galvanometer of high resistance. The stimulating electrodes were similar to those used in the nerve heat measurements and were at a similar distance from one another. The commutator, the condenser, the short circuit and series resistance, were all arranged as in the nerve heat measurements. A short stimulus was applied to the nerve (about 2 seconds) and the electric response read on a scale. In the upper half of fig. 5 the frequency was 51/sec. one-way shocks, in the lower half 229/sec.

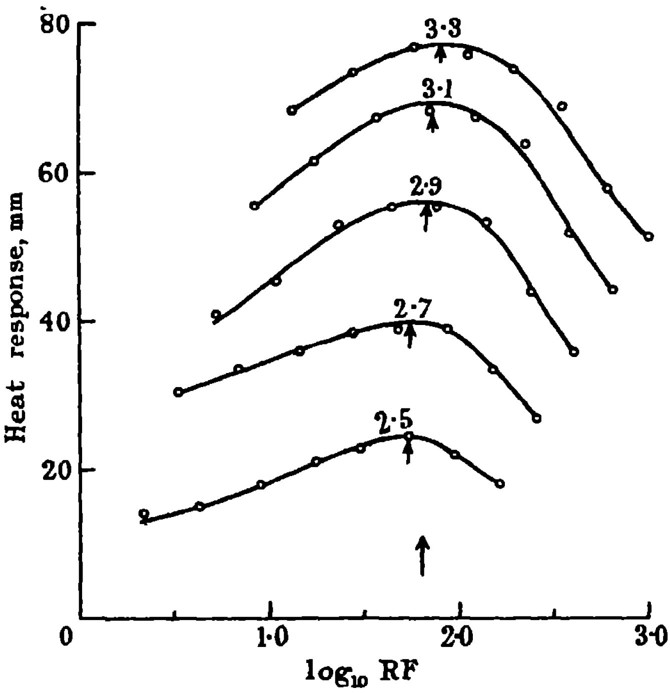

Fig. 4.

At the lower frequency eight values of the voltage were chosen from 0·8 to 12·7, and with each the capacity was varied so as to get responses above and below the chosen value of 50 mm. on the scale. The relation between response and log RF is shown in the figure. It is clearly identical in character with that obtained using the thermal response as an indicator. A line is ruled across the diagram at a height of 50 mm. and for the given response log RF can be read off for each voltage. Log V is plotted against log RF in fig. 6 (curve 1) and we see a relation exactly similar to that in figs. 2 and 3. For the higher frequency the response-log RF relation is given in the lower half of fig. 5. With the higher frequency the response was much bigger, and the upper part of each curve was not taken, so that the curves appear to be of a

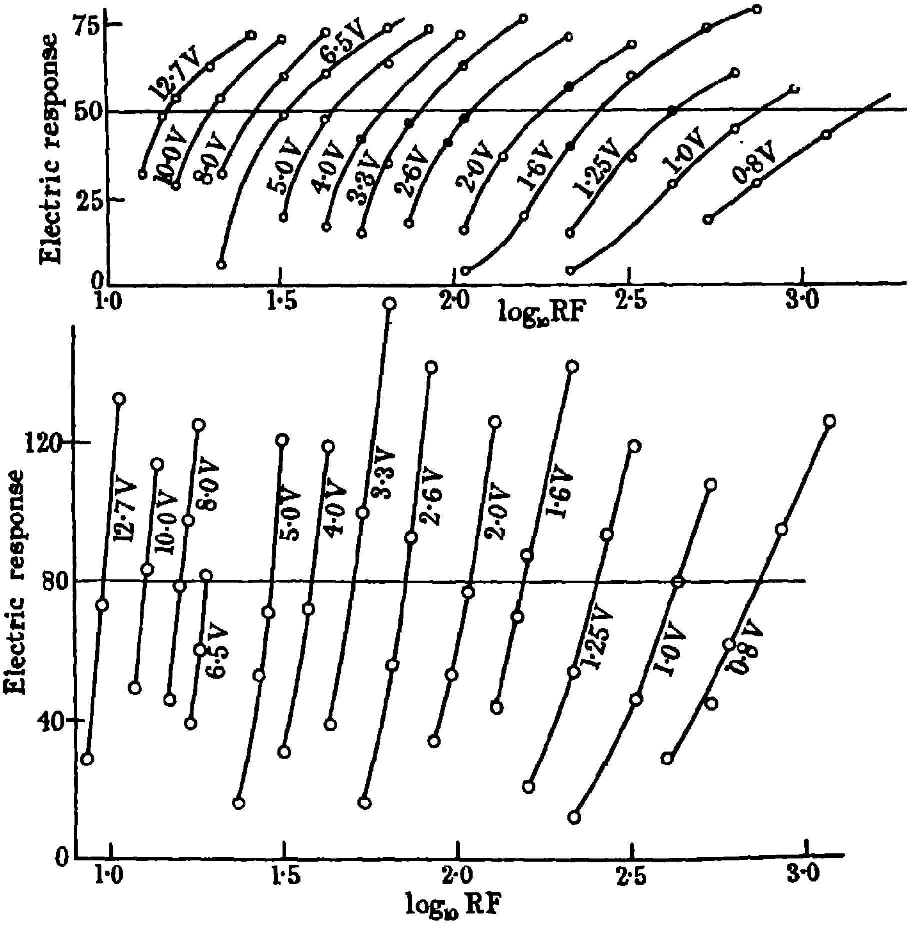

Fig. 5.

different shape. This is due simply to the fact that their tops are not included. Here again a line is ruled across the diagram at a height of 80 mm. and for the given response and each voltage the value of log RF is read off. In this way a log V-log RF relation is found which is plotted in fig. 6 (curve 2).

For these two curves the optimal stimuli can be read off at the two arrows. The mean for log RF is about 2·4; this is greater than for the heat measurements, partly at least because the temperature, 18·5° C., was considerably less.

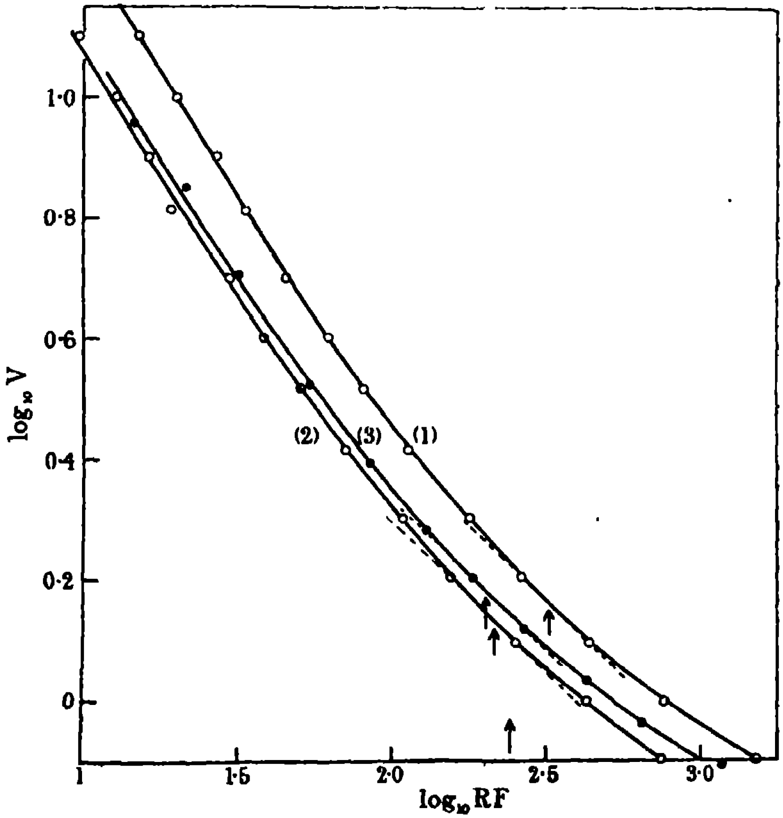

FIG. 6.

It now became evident that the same experiment could be performed on the electric change very much more quickly and simply by continuous stimulation. The commutator was set running and the condenser adjusted to a very small value. The voltage was then increased until, with the stimulus continuously running, a given size of the electric response was obtained on the scale. The capacity was changed and the voltage adjusted to give the same response. The capacity was again changed and again the voltage adjusted. In this way in a very *few minutes* a complete curve could be made, running over it from one end to the other and back, during continuous stimulation at any desired frequency. The method opens up possibilities which will be developed by Mr. Scott in a subsequentc ommunication. In fig. 6, curve 3 was obtained by this method, the nerve being stimulated with one-way shocks at 59/sec.; the optimal stimulus shown by the arrow is in the same region as that found by the other method.

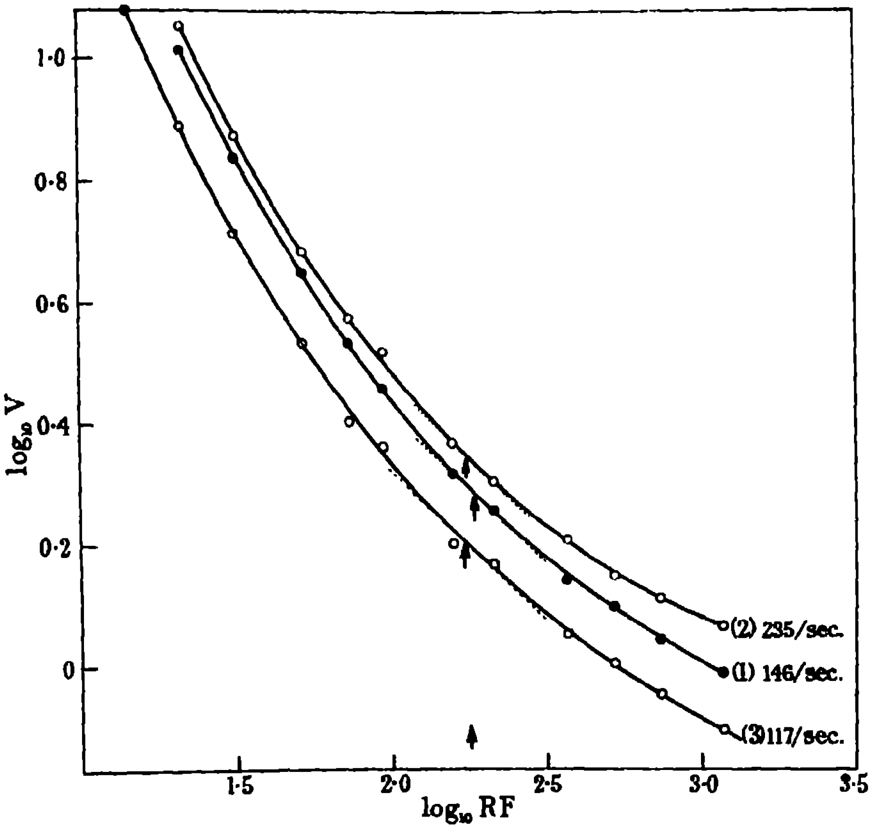

FIG. 7.

Fig. 7 shows another experiment made in the way just described. At about 18°, again with the assistance of Mr. Scott, the nerve was stimulated continuously at the frequency shown; the capacity was changed and the voltage was adjusted to give a constant deflection on the scale. The different curves are not for the same deflection and are not therefore directly comparable. The nerve had, in series with the stimulating electrodes, a resistance of 47,900 ohms, and across this and the electrodes was a shunt of 1087 ohms. The lowest curve was the mean of two series in opposite directions; the middle curve was the mean of four series, two in each direction, all taken in 30 minutes; the top curve was the mean of two series in opposite directions taken in 15 minutes. The optimal stimuli, determined as before with a parallel ruler, are in the neighbourhood of log RF = 2·25. This again is higher than in the heat production experiments owing to the lower temperature.

Finally an experiment was made on the mechanical response of a muscle stimulated with single condenser discharges. Owing to the rapid onset of fatigue continuous stimulation was not possible for muscle, but only single twitches. These were recorded by a photoelectric arrangement similar to that described by Kamada (1932). The area of the isometric twitch was read directly on a galvanometer scale, which is much more convenient than allowing the twitch to be recorded on smoked paper and then later reading it off. Here also the response is plotted in the upper part of the fig. 8 as a function of log RF. Eight voltages were chosen (0·7 to 5·02) and for each of these the capacity was given a series of different values so as to bracket the required response (30 mm. on the galvanometer scale). The experiment was carried out at about 23° C.; the nerve on the electrodes had 47,900 ohms in series with it, and the whole was shunted with 5010 ohms; a single condenser discharge was given by depressing a Morse key.

From the curves in the upper part of the figure the relation can be read off between log V and log RF for a given response in the same way as before. The upper curve in the lower half of the figure shows the result obtained; the lower curve was similarly obtained in another experiment at about the same temperature. The height of the curve depends upon such factors as the value of the series resistance and shunt. The form of the curve is a property of the nerve. The two curves are of the same shape as those found for the electric change and for the heat production. The optimal stimuli in the two curves are for log RF = 2·1 and 2·3 respectively.

The electric change and the heat production are recorded for all the fibres of the nerve, afferent and efferent alike; the mechanical response is recorded only for the motor fibres. The fact that the optimal stimulus for the twitch has a log RF slightly greater than for the heat production at nearly the same temperature may be due to this. In any case the divergence is comparatively small and there is no doubt that the form of the curve is the same.

Minor differences, therefore, there may be between the strength-duration curves obtained for the three different forms of responses. The fibres which give the greatest heat may not give the greatest electric change, nor may they be responsible for the muscular contraction. Each method of recording the response lumps all the fibres together with different weighting factors which may not be the same in the different types of response. There is no doubt, however, examining figs. 2 to 8 that the time characteristic of excitation when the thermal response is considered is similar to and almost quantitatively the same as that obtained by examining the electric change or the muscular response. If the heat production be an artefact it is in the same case as the mechanical response and the electrical response. One cannot conceive the possibility that the single relation found for all three in figs. 2 to 8 should be due to chance.

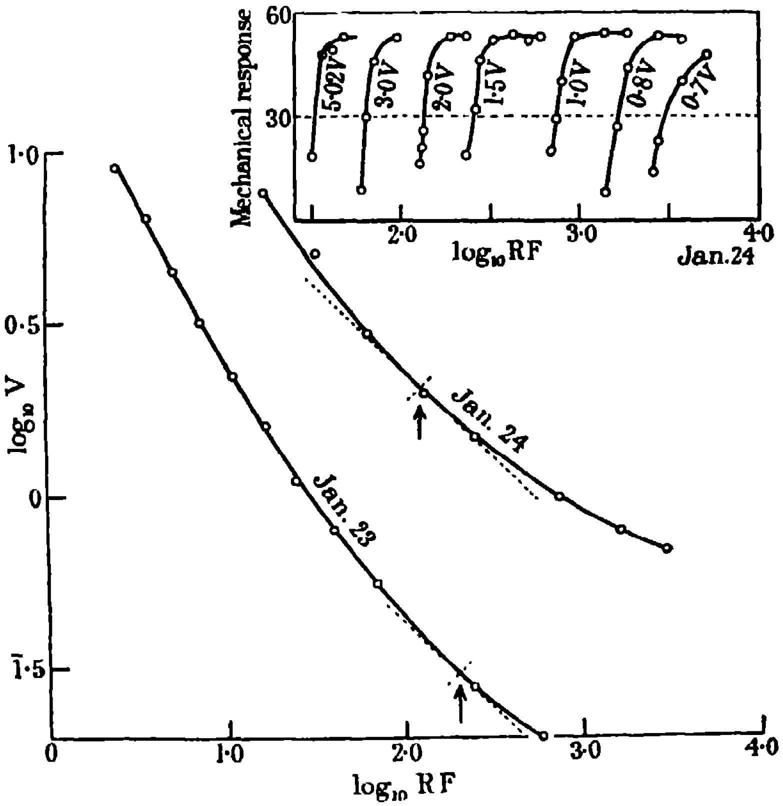

FIG. 8.

Summary.

(1) H. Winterstein has insisted that the oxygen consumption and the heat production found in electrically stimulated nerve are not due to a transmitted physiological effect (the impulse), but to a direct effect of the current on the nerve.

(2) The heat production is measured in a region of nerve 37 to 63 mm. distant from the stimulating electrodes, protected from current leak by an intermediate connection to earth. If current-spread were the cause of the observed heat it could only be in the outer fringe of a system of closed current lines round the electrodes, and the shocks would have to be enormously strong. In that case placing the electrodes nearer the section of nerve examined would greatly increase the effect. As a matter of fact, the heat is unchanged, and the relation between heat and strength of stimulus is unchanged when the distance of the electrodes is halved. The observed heat therefore is due to a transmitted and not to a direct effect.

(3) The electric change of nerve, and the mechanical response of muscle indirectly excited, are admittedly due to the transmitted impulse. They are normal physiological effects, the same whether stimulation be " natural " or " artificial." When they are produced " artificially " by electric excitation there is a well-known relation between the strength and the duration of the individual shocks. The " excitation time " and the " optimal stimulus " are well-defined quantities. The same relation exists, and the same quantities are found, for the heat production. It would be very strange if an electrical artefact had so characteristic a property in common with two known forms of physiological response.

(4) The heat production, therefore, like the electric change, is a normal property of the transmitted nervous impulse.

(5) A method is described by which the strength-duration relation for electric excitation can be determined for any nerve, motor or otherwise, during continuous stimulation at any frequency.

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The Action of Potassium and other Ions on the Injury Potential and Action Current in Maia *Nerve.*

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The electric currents in living tissues have interested physiologists ever since their existence was proved by Du Bois Reymond, but little was done towards explaining their origin and magnitude on a quantitative physicochemical basis before the pioneer researches of J. S. Macdonald. Work previous to Macdonald's has been discussed fully by that author (1902), hence it will not be mentioned here.

Macdonald found that the injury potential of medullated nerve, either frog's or mammal's (1900, *a*, *b*; 1902), could be given a value greater or smaller than the normal by simply altering the concentration of the solution in contact with the external surfaces of the fibres. He showed that solutions of NaOH, HCl, NaCl, or KCl affected the potential according to their concentrations and that the relationship between the two variables was a logarithmic one, which fact was in agreement with the then new theory of concentration cells developed by Nernst. Macdonald went on to point out (1902) that the effects

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* Part of the work described here was done during the tenure of a personal grant from the Medical Research Council.

of potassium chloride solutions were particularly interesting, and to suggest that the difference between the concentration of potassium in the axis cylinder of the nerve and in the surrounding blood or salt solution was wholly responsible for the observed injury potential. Over a wide range of concentration, 1/8 to 1 molar, he demonstrated that there was very nearly a linear relation between the injury potential and the logarithm of the potassium ion concentration. However, since he simply varied the concentrations of his electrolyte solutions, and made no effort to maintain them isotonic with blood by the addition of a non-electrolyte, exception might be taken to some of his results on the ground that the effects were due in part to the passage of water into or out of the nerve with a consequent dilution or concentration of the plasma.* Inspection of his results (1902) leaves little doubt that his experiments were complicated to some extent by such osmotic phenomena, and that his partial neglect of them led to the impossible value of 3½% when he attempted to calculate, by extrapolation, the concentration of the potassium chloride solution which would make the injury potential of mammalian nerve zero. Unfortunately these results led him to assume that such a concentrated solution must also exist in the axis cylinder of the nerve and many of his subsequent experiments (1902 and 1905) were concerned with locating more precisely this high salt concentration, and reconciling its existence with all that was already known about osmotic pressure.

Another difficulty in connection with Macdonald's experiments is that he did not state the condition of the nerves. There is no evidence that they were not seriously impaired, or even killed, by the soaking in the non-physiological solutions before the potential measurements were made.

Following Macdonald, Beutner (1912 onwards) has done a great deal to account for the magnitude and sense of bioelectric potentials, by his experiments and by the construction of models consisting of aqueous solutions separated by a membrane, or by a layer of a water-immiscible liquid. He has distinguished between two types of system (for references see 1920 and 1933), one with a symmetrical and one with an asymmetric membrane.

For an example of the first type we can cite the system :—

0·1 N KCl solution —	Salicylic aldehyde containing salicylic acid	0·0008 N KCl solution +

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* It might also have been expected that with the more concentrated potassium chloride solutions, Donnan distributions would affect the observed potentials appreciably. This question is discussed later.

which exhibits an e.m.f. of 0·10 volt. The measured e.m.f. is nearly equal to the maximum calculated according to Nernst's equation

$$E = \frac{RT}{nF} \log_e C_1/C_2,$$

which at 18° C. becomes

$$E = 0{\cdot}058 \log_{10} C_1/C_2,$$

approximately 0·12 volt, when allowance has been made for the difference between concentrations and "activities" (Lewis and Randall, 1923, chap. 27) in the aqueous phases. If, for the slightly acidic water-immiscible phase, a slightly basic and still water-immiscible substance toluidine is substituted then the e.m.f. of the cell is not only reduced to about one-half of its former value but also its sense is reversed.

A system of the second type, analogous to Cremer's nitrobenzene chain, is the following double membrane cell :—

+			−
Calomel electrode with 0·1 N KCl	Cresol	Benzaldehyde	Calomel electrode with 0·1 N KCl

When the two aqueous phases are identical the e.m.f. is 0·13 volt.

Besides the two types of cell described by Beutner a third type is possible in which the asymmetry is due to the presence of a non-diffusible ion on one side of a membrane separating two electrolyte solutions. The behaviour of such systems is embraced in the Donnan theory of membrane equilibria (1911).

Whilst Macdonald (1900, *a* and *b* ; 1902) seems to have favoured the view that medullated nerve is bounded by a symmetrical membrane his experiments are inconclusive, and even if it were possible to avoid the internal short circuiting, which occurs so much more easily with animal tissues than with plant cells, e.m.f. measurements would still be inconclusive without knowledge of the chemical constitution of the axis cylinder.

From the observations of Beutner, Osterhout, and other workers it would seem that the membranes bounding living cells are more commonly asymmetric than symmetrical. Beutner (for references see 1920 and 1933) has studied apple peel as an example of a membrane of the asymmetric type, and more recently Osterhout and his collaborators (for references see Osterhout, 1931) have shown that the membranes enveloping large single plant cells of *Nitella clavata* and *Valonia macrophysa* are asymmetric. With *Nitella* both the concentration effect and the asymmetric effect have been investigated on the same membrane. The arrangement

+		−
sap (interior)	protoplasmic wall	sap (exterior)

gives a potential difference of about 15 millivolts, and if potassium chloride solutions of increasing strength are applied in succession to the exterior of the cell the "injury" potential decreases in steps to zero, and with a further increase in the potassium chloride concentration a potential in the opposite sense is obtained.

As non-medullated nerve is so much simpler in structure than medullated nerve, and the heat production and electrical changes are so much greater, Professor A. V. Hill suggested that it would be worth trying to correlate the "depolarization" of crab's nerve produced by asphyxia or stimulation (Furusawa, 1929) with any accompanying chemical changes which might be detected. The work was begun by examining the inorganic ions contained in nerve: preliminary analyses showed that potassium is the only cation besides sodium present in any considerable quantity and, keeping in mind the data summarized above, an attempt to connect this fact with nerve function led to the more physiological experiments described in the other sections of this paper.

The Estimation of Potassium and other Substances in Crab's Blood and Nerve.

Because in January and February, 1932, specimens of *Maia squinado* were not readily available, some preliminary analyses were made with samples of blood serum and nerve which had been obtained from five living specimens of *Cancer pagurus* at the Marine Biological Laboratory, Plymouth, and sent to London in glass stoppered bottles. Other samples of *Cancer* blood serum and nerve were obtained from two living specimens supplied by a local fishmonger. The serum was always poured off from the clot after the freshly drawn blood had been allowed to stand for about 15 minutes.

Blood serum samples of approximately 10 c.c. were weighed into silica crucibles and their water contents were determined by drying at 105° C. until constant weights of residue were obtained. After the residue had been charred with concentrated sulphuric acid, and nitric acid had been added until all organic matter was completely oxidized, a trace of ammonium carbonate was added, the material was heated to dull redness, then cooled and weighed as cation sulphates. These were dissolved in hot 0·1 N acetic acid and the potassium present was estimated gravimetrically as the sodium potassium cobaltinitrite according to the method described in Treadwell and Hall (1924). As the composition of the precipitate varies somewhat under different conditions an empirical factor was found by employing a solution containing "analytical reagent" sodium and potassium chlorides in the amounts in which they occur in sea water. The analyses were further checked by determining the potassium content of Plymouth "outside sea water," which had been collected at the international hydrographical station E.1. The results were in agreement with those obtained by Dittmar (1884) for Atlantic sea water.

With seven specimens of *Cancer* blood the differences between duplicate determinations of total solids, inorganic sulphates, and potassium were less than 1% of the amount of substance estimated. The extreme values for the total solid content of different specimens were 6·8 and 11·7%, and the mean value is 9·7%, when the one low result 6·8% is excluded. In spite of these variations in the solid content of different specimens the ratio of the total inorganic sulphate residue to the water present was very constant. The potassium contents of the samples were also fairly constant, lying between 46 and 55 mg. per 100 gm. of water (Table I).

The nerve samples, which had been blotted carefully on "gravimetric" filter paper (Whatman, 42 which is free from fluff and soluble organic matter), were placed in silica crucibles, weighed, and examined in the same manner as the blood samples. The results of duplicate analyses, which also are summarized in Table I, were rather less consistent than for blood, owing to the difficulties of isolating nerve free from connective tissue, and on account of the variable amounts of water retained, even after careful blotting, between the secondary bundles constituting the nerve trunk.

Four 10-c.c. specimens of *Maia* blood serum were also examined. The total solid and total inorganic cation sulphate contents were similar to those occurring in *Cancer* blood whilst the potassium content was slightly lower. Two samples of *Maia* nerve had approximately the same potassium content as *Cancer* nerve, Table II. The water contents of the *Maia* nerves were found to be about 1% less than those of the nerves used by Meyerhof and Schulz (1929)—probably this is due to the differences in the method of blotting and drying.

Table I.

Cancer Blood Serum.

Specimen.	Total solids per 100 gm. of serum.	Cations as sulphates per 100 gm. of serum.	Cations as sulphates per 100 gm. of water.	Potassium per 100 gm. of serum.	Potassium per 100 gm. of water.
A	6·80	4·01	4·29	0·0480	0·0515
B	10·31	3·84	4·28	0·0450	0·0502
C	11·69	3·81	4·31	0·0432	0·0489
D	9·48	3·88	4·29	0·0502	0·0554
E	8·28	3·94	4·32	0·0426	0·0464
F	8·84	3·92	4·28	0·0505	0·0554
G	9·71	3·90	4·35	0·0446	0·0494

Sea Water.

Specimen.	Total solids per 100 gm. of solution.	Cations as sulphates per 100 gm. of solution.	Cations as sulphates per 100 gm. of water.	Potassium per 100 gm. of solution.	Potassium per 100 gm. of water.
a	3·45	4·17	4·33	0·0392	0·0406
b	3·47	4·22	4·35	0·0387	0·0402

Cancer Nerve.

Specimen.	Total solids per 100 gm. of nerve.	Cations as sulphates per 100 gm. of nerve.	Cations as sulphates per 100 gm. of water.	Potassium per 100 gm. of nerve	Potassium per 100 gm. of water.
A	14·22 13·96	3·71 3·62	4·33 4·22	0·503 0·526	0·581 0·611
B	14·31 14·12	3·79 3·72	4·42 4·34	0·491 0·503	0·573 0·585
C	13·84 14·07	3·78 3·68	4·42 4·30	0·557 0·564	0·646 0·657
D	14·21 14·52	3·79 3·84	4·38 4·44	0·534 0·527	0·623 0·617

As the methods described above for the determination of potassium were rather tedious, and required relatively large amounts of material, it was decided to adopt the method described by Kramer (1920) for 1 c.c. samples of human serum. It was, however, modified, so as to increase the accuracy and reliability, and enable it to be used to measure the rate of leakage of potassium from stimulated nerves (see later).

Table II.

Maia Blood Serum.

Specimen.	Total solid per 100 gm. of serum.	Cations as sulphates per 100 gm. of serum.	Cations as sulphates per 100 gm. of water.	Potassium per 100 gm. of serum.	Potassium per 100 gm. of water.
A	10·50	3·83	4·29	0·0360	0·0403
B	8·37	3·90	4·32	0·0392	0·0428
C	8·59	3·93	4·30	0·0413	0·0452
D	9·38	3·89	4·30	0·0389	0·0429

Maia Nerve.

Specimen.	Total solid per 100 gm. of nerve.	Cations as sulphates per 100 gm. of nerve.	Cations as sulphates per 100 gm. of water.	Potassium per 100 gm. of nerve.	Potassium per 100 gm. of water.
A	13·13	3·78	4·34	0·528	0·615
	13·30	3·70	4·26	0·520	0·600
B	13·27	3·73	4·28	0·508	0·585
	13·10	3·79	4·35	0·514	0·502

The modifications were :—

(*a*) At least 12 hours were allowed for the formation of the cobaltinitrite precipitate since it is not really complete in half an hour.

(*b*) The adoption of a smaller Gooch crucible and the reduction in the amount of asbestos fibre used. This meant that it was unnecessary to pick out the asbestos pad and drop it into the acidified potassium permanganate solution; instead the Gooch crucible (1 c.c. capacity) and its contents could be dropped in, eliminating the transfer, a source of error since the precipitate floated to the sides unless great care was taken.

(*c*) The precipitate was washed with 0·1 N acetic acid, in which it is less soluble than in water; about 12 c.c. divided into four portions was used to wash the amount containing 0·3 mg. of potassium.

(*d*) Instead of 0·02 N. potassium permanganate and 0·01 N oxalic acid, 0·1 N solutions were used. The merit of this was that for the titration the total volume of solution was reduced from about 30 c.c. to 3 c.c. and the uncertainty of the end point was reduced from 0·05 c.c. of 0·01 N potassium permanganate solution to 0·001 c.c. of 0·1 N permanganate solution. Rehberg (1925) has pointed out that the use of small amounts of concentrated solutions would lead to such an improvement.

(*e*) To gain the advantages mentioned in (*d*) pipetting errors were eliminated by the use of the syringe pipettes described by Krogh and Keys (1931). These instruments can be adjusted to deliver volumes of about 1 c.c. with a reproducibility of 1 in 10,000. Instead of a burette a micrometer syringe similar to that described by Trevan (1925) was used, but modified so that temperature variations were approximately compensated in the same manner as in the syringe pipettes. The syringe pipettes and the burette were fitted with glass needles, the ends of which had been drawn out to fine jets. I am indebted to Dr. A. Keys for his advice about the use of these.

With the modifications described above the consistency with which 1 c.c. samples of approximately the same concentration could be compared was to within about ± 0·5%, but the absolute accuracy of a duplicate determination was only to within ± 1% because of the uncertainty of the exact composition of the precipitate when it is formed under slightly different conditions. For this reason, whenever possible, the determination with the unknown solution was checked with a known solution of approximately the same concentration.

The procedure was designed for 1 c.c. of crab's blood serum, or 50 mg. of nerve, which amounts could be obtained from a single walking leg of a medium sized specimen of *Maia*. The limb was cut off and the blood from it, except for the first few drops, was drained into a small flask which was corked and shaken until a clot had formed. Then two or three samples were measured into silica test tubes by means of a syringe pipette, which had been adjusted to deliver exactly 1 c.c. The nerve was dissected out (see next section), washed in sea water, blotted on " gravimetric " filter paper, put into a weighing bottle, weighed and transferred, along with the washings from the weighing bottle, to a silica test tube. Batches of specimens so prepared were dryed in an oven, small soda glass funnels were then placed in the mouths of the test tubes to prevent loss of material whilst fuming nitric acid was dropped in. When the oxidation was finished the fluid on the funnels was rinsed into the test tubes with a few drops of distilled water and the contents were evaporated to dryness. One drop of concentrated sulphuric acid was added to each, the tubes were heated almost to redness to remove any excess of acid, and allowed to cool, each residue was then dissolved in 1 c.c. of 0·1 N acetic acid, and transferred with the aid of a few drops of distilled water to a small beaker. One c.c. of 20% "analytical reagent" sodium cobaltinitrite in 0·1 N acetic acid was added to each sample, slowly, drop by drop, with continual shaking.

After the specimens had stood overnight the determinations were made according to the following details. By means of a syringe pipette a volume of approximately 0·1 N potassium permanganate solution exactly equivalent to 1 c.c. of 0·1 N oxalic acid solution was measured into a small wide-mouthed conical flask and 1 c.c. of 4 N sulphuric acid was added. Then a Gooch crucible was prepared with a pad of asbestos about 1 mm. thick and washed with 0·1 N acetic acid. The cobaltinitrite precipitate from one of the samples was filtered off and washed carefully with the measured amount of 0·1 N acetic acid. The Gooch and its contents were dropped into the measured volume of acidified permanganate solution; the flask was shaken until the asbestos pad had disintegrated and then it was placed in a boiling water bath for 1 minute. At the end of that time 1 c.c. of 0·1 N oxalic acid was added from a syringe pipette and the heating was continued until the suspension had become quite colourless. Finally, the excess of oxalic acid was measured by a back titration with more of the approximately 0·1 N permanganate solution from the micrometer syringe.

The controls with solutions of known potassium content were done at the beginning and end of each series of determinations, also blanks with the Gooch crucibles and asbestos.

For illustration the protocol of a duplicate determination of the potassium in 1 c.c. samples of *Maia* blood is given. In Table III are the results for the potassium contents of specimens of sea water, *Maia* blood and nerve taken in September, 1932, and November, 1933. They are all expressed in grams per 100 gm. of fluid, since the specific gravities of many of the samples were determined by weighing them.

From these analyses it is evident that in each 100 gm. of water in *Maia* nerve there is about 13 times as much potassium as in each 100 gm. of water in the blood. The true ratio may well be higher if an appreciable quantity of sea water is retained between the bundles of fibres constituting the nerve trunk. Also, attention may be drawn to the analyses of *Maia* blood by Leulier and Bernard (1931). They found 0·0486% of potassium in Mediterranean sea water, and 0·0752% in *Maia* blood. As their values were not in agreement with those obtained here the experiments of November, 1933, were made to check the previous ones. It may be that the differences are due to some environmental factor.

Table III.

Maia Blood Serum and Nerve.

September, 1932.		Crab.	November, 1933.	
Potassium per 100 gm. of blood.	Potassium per 100 gm. of nerve.		Potassium per 100 gm. of blood.	Potassium per 100 gm. of nerve.
0·0423	0·519	A	0·0431	0·529
0·0392	0·516	B	0·0408	0·418
0·0374	0·436	C	0·0382	0·429
0·0408	0·545	D	0·0394	0·517
0·0433	0·478	E	0·0402	0·497
0·0418	0·503	F	0·0413	0·538

Plymouth " Outside Sea Water."

Potassium per 100 gm. of sea water.
0·0389
0·0388
0·0385
0·0387

Specific gravities of 1 c.c. samples of *Maia* blood at 12·1° C. : 1·0406, 1·0423, 1·0418, 1·0402.
Specific gravity of sea water at 12·1° C. : 1·024.

Protocol of Duplicate Determination of Potassium in Maia *Blood Serum.*

For analysis : Two 1 c.c. samples from specimen C of *Maia* blood serum, November, 1933.
Comparison solutions : Three 1 c.c. samples of an artificial sea water containing 0·040 gm. of potassium per 100 c.c. of solution.

The blood samples were ashed and the cobaltinitrite reagent was added to acetic acid solutions of the ash, and to the sea water samples. The titrations were performed the following morning. The volumes of permanganate solution required for the back titrations are given below.

Artificial sea water—

$$\left.\begin{array}{l} 5{\cdot}79 \times 0{\cdot}0970 = 0{\cdot}562 \text{ c.c.} \\ 5{\cdot}76 \times 0{\cdot}0970 = 0{\cdot}559 \text{ c.c.} \\ 5{\cdot}78 \times 0{\cdot}0970 = 0{\cdot}560 \text{ c.c.} \end{array}\right\} \text{Mean } 0{\cdot}560 \text{ c.c.}$$

Maia *serum*—

$$\left.\begin{array}{l} 5{\cdot}72 \times 0{\cdot}0970 = 0{\cdot}555 \text{ c.c.} \\ 5{\cdot}77 \times 0{\cdot}0970 = 0{\cdot}559 \text{ c.c.} \end{array}\right\} \text{Mean } 0{\cdot}557 \text{ c.c.}$$

Blanks, with Gooch crucible and asbestos but no precipitate—

$$\left.\begin{array}{l} 0{\cdot}31 \times 0{\cdot}0970 = 0{\cdot}030 \text{ c.c.} \\ 0{\cdot}32 \times 0{\cdot}0970 = 0{\cdot}031 \text{ c.c.} \end{array}\right\} \text{Mean } 0{\cdot}031 \text{ c.c.}$$

Specific gravity of 1 c.c. sample of *Maia* serum : 1·0402.

$$100 \text{ gm. of } \textit{Maia} \text{ serum contain } \frac{0{\cdot}040 \;\; (0{\cdot}557 - 0{\cdot}031)}{1{\cdot}0402\,(0{\cdot}560 - 0{\cdot}031)} \text{ gm. of potassium}$$

$$= 0{\cdot}0382 \text{ gm. of potassium.}$$

The Effect of Potassium and other Ions on the Injury and Action Potentials.

Method.—For the physiological experiments nerves from the walking legs of large specimens of *Maia squinado*, which were taken from the Plymouth storage tanks and were neither about to moult nor had just moulted, were dissected carefully in the manner described by Levin (1927), but no attempt was made to free them completely from connective tissue as this would have involved the risk of injury. The nerve was tied at the ends and suspended in aerated sea water for about an hour to prevent the reversible inexcitability described by Furusawa (1929); it was then mounted in a paraffin wax chamber on the floor of which was sea water to ensure against drying, the peripheral end was crushed and the chamber closed by a glass cover sealed on with plasticene. Oxygen was passed first through a wash bottle containing sea water, and then through the nerve chamber at the rate of about ½ c.c. per second.

The monophasic action current was led off by two strips of filter paper soaked in sea water to small reservoirs of sea water into which dipped the capillary tubes from calomel half cells, which had also been made up with sea water; from these it went through a variable shunt to a moving coil galvanometer. For the first few weeks a Cambridge A and M (short suspension) instrument of 450 ohms resistance and period 7 seconds was used, but a shunt of 2500 ohms in addition to the nerve and calomel electrodes was necessary for critical damping, with the result that a good deal of sensitivity was lost. For the greater part of the time a special Cambridge A and M instrument, critically damped, of 1000 ohms resistance, period 3·8 second, and sensitivity 1 mm. = $4 \cdot 10^{-10}$ amp. at 3 metres, was employed.

The spot was always perfectly steady and its position could be read to 0·1 mm. The temperature in the cellar rarely rose above 17° C. in the summer and in winter this temperature was maintained within ± 0·5° C. by switching on and off an electric radiator.

In the nerve chamber were two pairs of silver-wire electrodes so that the place of application of the stimulus could be varied when it was desired to avoid the local fatigue described by Levin (1927). Between these electrodes and the action current leads was placed an additional silver-wire electrode, connected to earth to obviate leakage of the stimulating current into the galvanometer circuit.

In the preliminary experiments stimuli were obtained from a Harvard coil with the secondary fixed at 9, 2 volts in the primary, and frequencies varying from 20 make and break to 70 make and break shocks per second were achieved by loading the hammer of the interruptor with plasticene. However, as Levin (1927) pointed out, the make shocks are almost ineffective but the break shocks are probably supra-maximal, and Furusawa's (1929, p. 332) example confirms that it is impossible to increase these stimuli owing to the ease with which local fatigue is produced. The unsuitability of such stimuli is further emphasized by the work of Beresina and Feng (1933) on the heat production of *Maia* nerve; consequently, it was decided to employ repetitive condenser stimulation with their value of "optimal stimulus." Using a shunt of 1500 ohms and a series resistance of 3000 ohms to render the condenser (0·95 μF) discharge time approximately independent of the resistance of the nerve, just maximal stimuli were provided by a rotating commutator; usually a frequency of 49 per second was used and the condensers were charged to a potential of 14–18 volts.

Previous to mounting the nerve, fresh filter paper strips, each 35 mm. by 7 mm., soaked in sea water and drained of all surplus fluid were placed in position and another strip placed across them to complete the circuit; any small e.m.f. between the calomel half cells was measured, its direction noted and the correction applied to subsequent readings. The nerve was mounted and during the first few minutes after the crushing of the end the galvanometer deflection rose to a maximum, then declined at a decreasing rate: the maximum value was taken to be the injury potential of the nerve. After the spot of light had become fairly steady, or was falling at a constant speed, a short circuiting key was opened and an e.m.f. from a Pye rotary potential divider in series with the nerve, fig. 1, was adjusted to bring the spot to zero and then the deflection produced by a known change in e.m.f. was observed. The short circuiting key was closed and the deflection due to the nerve was again read; if it had changed only slightly an allowance was made, but if the change was large the measurement was repeated later when the drift of the galvanometer spot had become smaller. From the sensitivity determined in this way the injury potential corresponding to the maximum deflection was calculated. The method, just described, of measuring the injury potential was used because immediately after the injury had been made the galvanometer spot was moving too rapidly to permit measurements to be made by the ordinary compensation method.

For each measurement the galvanometer was suitably shunted to reduce the deflection to 150–200 mm. and readings could be made to 0·1 mm., whilst e.m.f. applied from the Pye rotary potential divider could be adjusted to 1 in 10,000. The resultant possible accuracy of a single determination was therefore about ± 1 in 1000 or ± 0·03 millivolt, but this was never needed since even in successive measurements on the same nerve such a consistency was never approached, because various factors which are discussed later combined to limit the reproducibility to ± 5% of the initial injury potential.

Usually the nerve was given a 3-second test tetanus shortly after the deflection due to the injury potential had passed through its maximum, another about 10 minutes later, a third tetanus after the sensitivity had been determined by introducing a known e.m.f. into the circuit, and further tetani were given at intervals of 10 minutes or more if required. Most of the measurements of action current and injury potential were made with the same shunt in the galvanometer circuit, but if it was desired to make the deflection due to the action current larger the injury potential was partly balanced by an e.m.f. from the Pye rotary divider and then the shunt was reduced: in this way deflections as great as 200 mm. were obtained for the response to a 3-second tetanus, frequency 49 per second.

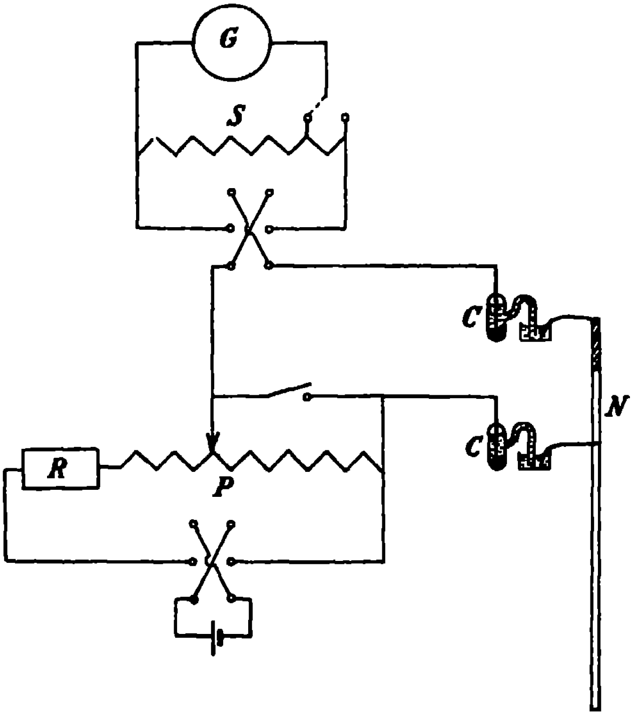


FIG. 1.—G, galvanometer; S, variable shunt; R, fixed resistance, 1000 ohms; P, rotary potential divider, total resistance 28 ohms; C, calomel half cells; N, nerve, crushed part shaded.

The first series of experiments was made with "sea water" rich in potassium or calcium or in both potassium and calcium. Solutions of potassium chloride and calcium chloride calculated to be isotonic with Plymouth "outside sea water" (hereinafter called sea water) were prepared with chemicals of

" analytical reagent " quality and distilled water, which was known to contain the heavy metals, copper and tin, in smaller amounts than does sea water (Atkins, 1932, 1933, *a*, *b*), and the required volumes were added to sea water. The p_H of these enriched sea waters was found to be that of ordinary sea water 8·1–8·2 when determinations were made with brom-thymol blue (Atkins, 1922), the salt error being eliminated by the use of McClendon's (1917) borax boric acid buffer standards made up with salt solution.

Owing to the fact that the addition of isotonic potassium chloride solution altered the concentration of the other ions, besides sodium, in sea water the experiments were repeated and checked with artificial sea waters in which the potassium, calcium, and magnesium concentrations were varied independently or in combination with one another. Simultaneously a corresponding adjustment was made in the sodium chloride content so that the solutions were always as nearly as possible isotonic with 3·30% sodium chloride solution, which is known to be isotonic with sea water, and since *Maia* has no power to regulate its osmotic pressure and comes into equilibrium with its environment (Margaria, 1931) it follows that they were also isotonic with *Maia's* blood and nerve. The solutions were prepared by dissolving the required amounts (calculated from Clark, 1924; Harvey, 1928) of sodium chloride, sodium bromide, sodium carbonate, sodium bicarbonate, potassium chloride, magnesium chloride, and calcium chloride, all of " analytical reagent " quality in distilled water. The carbonate and bicarbonate contents of the solutions were always kept the same in order that they should all have the same p_H and alkali reserve. After they had been brought into equilibrium with atmospheric air at 17° C. by vigorous aeration the p_H values were found to lie between 8·1 and 8·2.

In order to investigate the action of other cations which do not occur in any considerable quantity in sea water isotonic solutions of lithium chloride, rubidium chloride, cæsium chloride, and ammonium chloride, were made up with substances of " analytical reagent " quality, in distilled water and used to prepare " sea waters " rich in these ions. When it was desired to investigate the physiological antagonism existing between the alkali metal ions lithium, rubidium, and cæsium on the one hand, and calcium ions on the other, known quantities of isotonic calcium chloride solution were also added. All the solutions had the same p_H as untreated sea water.

In order to be certain that the results which had been obtained with the enriched " sea waters " were independent of the presence of the potassium, which occurs in ordinary sea water, a second series of artificial sea waters containing varying amounts of lithium, rubidium, or cæsium, but no potassium, was prepared. These solutions were also isotonic with 3·30% sodium chloride solution, and had a p_H of 8·1–8·2 when in equilibrium with atmospheric air.

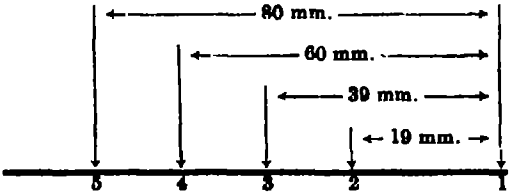
For nerve asphyxiation experiments cylinder nitrogen was freed from oxygen by passage through two wash bottles filled with alkaline hydrosulphite solution (Kautsky and Thiele, 1926), through a wash bottle containing 40% caustic potash solution, and then through a bubbler of sea water; this last being to ensure that the nerve was not dried.

The Injury Potential of "Normal" Nerve.

Soon after experiments at Plymouth had been begun it was noted that the injury potentials observed were greater than those recorded by Levin (1927) and by Furusawa (1929) although all the measurements were made by very similar methods. Levin reported injury potentials of 10–13 millivolts in *Maia* nerve, and Furusawa reported values usually ranging from 2·5 to 13 millivolts and a highest value of 27·3 millivolts with nerves, not dissected, but prepared by the "pulling out" technique which he described. In the experiments recorded here the most usual value of the injury potential of a nerve, which had been dissected by Levin's method with the precautions already mentioned and allowed to rest in aerated sea water for an hour, was about 30 millivolts although values ranging from 10 to 35 millivolts were observed and on one occasion a value of 42·1 millivolts was obtained. As the injury potentials were larger than those previously reported it was thought advisable to investigate the factors governing their magnitude and reproducibility. Moreover, it was desired to know how long the potential would remain constant and how well *Maia* nerve would stand removal from the wax chamber, washing and re-mounting, when these operations were repeated three or four times.

The influence of the method of preparation was one of the factors first investigated. It was found that different nerves taken from one crab and prepared by Levin's method, gave injury potentials which were the same within the experimental error and this fact has been regarded as justifying the experiments following. Four nerves, which were prepared by Furusawa's (1929) method (without washing in sea water) from four different crabs gave injury potentials of 5–9 millivolts, but variations of 2–3 millivolts were observed when the filter paper electrode making contact with the uninjured part of the nerve was moved along the trunk, care being taken not to disturb the injured end. Four other nerves, which were taken from the same four crabs and prepared by Levin's method, gave injury potentials of about 30 millivolts, and when the electrode making contact with the uninjured part of the trunk was moved the variations were usually of the order of 1 millivolt, although occasionally they were as large as 2 millivolts, Table IV. From these experiments it is concluded that Furusawa's method usually gives low injury potentials, probably because many of the fibres near the surface of the bundle are injured; this view is supported to some extent by the observation of potential differences, which formed a fairly large fraction of the whole injury potential, between various points of the nerve trunk. Preparation by Levin's method with the precautions already described seemed to be preferable although the trunk was not obtained wholly free from small injuries which had some influence on the reproducibility of the injury potential if between successive determinations it was necessary to disturb the uninjured part of the nerve lying on the leading off electrode.

Table IV.—Four determinations were made with each nerve, and allowance was made for the apparent decrease of the injury potential with time. The distances between the leading off electrodes are indicated in the diagram.



Crab.	Nerve prepared by Furusawa's method.				Nerve prepared by Levin's method.			
	E.m.f. in millivolts between electrodes:				E.m.f. in millivolts between electrodes:			
	1 and 2.	1 and 3.	1 and 4.	1 and 5.	1 and 2.	1 and 3.	1 and 4.	1 and 5.
K	5·4	6·9	6·2	5·2	27·6	26·4	26·2	27·4
M	6·3	5·7	4·6	7·8	28·6	28·4	28·8	29·9
Q	6·0	7·8	8·4	9·1	32·1	31·4	30·6	31·2
S	7·6	8·2	8·4	—	33·0	32·1	31·7	33·7

The next important factor was found to be the sharpness of the boundary between the injured and uninjured portions of the nerve. There are really two variables involved and to a limited extent they are mutually dependant upon one another; the first is the sharpness of the initial demarcation and the second is the change in the demarcation with time.

With respect to the first of the variables it was found that the procedure which gave the highest injury potential was to make a block by crushing transversely with small forceps at a point about 2 mm. from the distal leading off electrode, and then to crush completely the peripheral part of the nerve beyond the initial injury. If, however, a ligature was tied midway between the galvanometer electrodes and then the peripheral part of the nerve beyond the block was crushed, the injury potential observed was only 80% or less of that obtained by the former method. It seems very probable that the lower value given by the latter procedure was due to a failure to make proper contact with the inside solution. A few attempts were made to dry the outside surface of the nerve, by blotting with filter paper, in the hope that there would be less short circuiting and that a higher injury potential would be attained; but they were not successful because if, on the one hand, the filter paper was only applied lightly the sea water was not removed completely from between the bundles, if, on the other hand, it was applied more firmly the nerve stuck to it and some fibres were damaged. In order to reduce the uncertainties just discussed to a minimum and to obtain the maximum injury potential a standard procedure of simply draining the nerve as free as possible from surplus fluid and then crushing exactly in the manner first described above was adopted. As an additional precaution against dead tissue at the peripheral end of the nerve becoming permeated with sea water and affecting the injury potential the last 5 mm. were cut off each time immediately before the mounting.

Experiments similar to those just recorded have since been made with frog's nerve and the effects observed were greater. The injury potential of a nerve which had been ligatured between the leading off electrodes and had its end crushed was only about 60% of that given by a nerve which had merely had its end crushed with forceps.

The operation of the second variable was observed by Levin (1927), but neither he nor Furusawa seems to have realized exactly what was happening. Both of these investigators noted that the galvanometer deflection rose and passed through a maximum within a short time of the crushing of the end of the nerve, and then it fell at a steady or decreasing rate. They assumed that the decline in the deflection was due to a diminution in the injury potential, whereas it is really a "crush sealing over" effect similar in some respects to that observed on frog's nerve by Gerard (1930). This was shown in the following way: the fall of the galvanometer deflection was observed for half an hour or more after the initial value of the injury potential had been determined and then if a fresh crush was made, with a small pair of forceps, to restore the sharpness of the boundary between the injured and uninjured parts, care being taken not to disturb the uninjured nerve lying on the other galvanometer electrode, the deflection rose again and a second value of injury potential which was usually within ± 5% of the first was obtained. However, if the second crush was merely made within the region which had been previously injured there was no increase in the galvanometer deflection.

In a nerve, which had been dissected by Levin's method and soaked for an hour in aerated sea water, when suspended in moist oxygen and re-mounted on fresh pieces of filter paper at half-hourly intervals the injury potential was reproducible to within ± 5% of its value, provided that a fresh crush was made each time and the precautions detailed were observed. At 17° C., under the conditions just specified, and when stimulation was limited to occasional test tetani of 3 seconds duration this reproducibility of injury potential persisted for 2–3 hours, but at the end of about 8 hours there was a drop of 20–30% corresponding to a steady decline of about 3% per hour. If, in view of Furusawa's work (1929) on the oxidative nature of the injury potential of *Maia* nerve, we assume that the potential is exactly paralleled by the oxygen consumption (see also Lund on the potential across frog's skin, 1928) then such a figure for the rate of decline of the injury potential is not unreasonable. Chang (1931) has found that the oxygen consumption of the leg nerves of *Homarus americanus* falls at about 10% per hour for the first 4 hours at 24° C., and if we take Q_{10} to be 2·5 the rate of fall at 17° C. would be 4·2% per hour.

Transference to aerated sea water between successive determinations had no effect on the nerve when the mounting process was not repeated more than once in about 20 minutes: if it was done more frequently the attendant handling and crushing caused a partial "depolarization," and low values of injury potential were obtained.

There was no sign of any seasonal variation in the injury potential of *Maia* nerve; during the period mid-April to mid-December the usual value was 30 millivolts. Another possibility, that captivity in the Plymouth storage tanks might have had some influence, was negatived by keeping eight marked crabs (4 male and 4 female) in the tanks from the middle of April to the end of May—a longer period than that for which the specimens used in the experiments described here were kept. Periodically a leg was removed and the nerve was dissected out: there was no sign of any progressive change in the injury potential; with nerves from the same crab, a variation greater than the ordinary experimental error was never observed.

The Action of Potassium Rich and Potassium Deficient Solutions on the Injury Potential and Action Current.

Method.—The action current, the retention of action current and the depolarization of crab's nerve have been studied in some detail by Levin (1927) and by Furusawa (1929). Both observers used rapid Downing galvanometers which were more suitable for the purpose than the slower instruments employed in these experiments, in which the main concern was the injury potential and the action current was used only as an indication of the physiological condition of the nerve.

After its action current had been tested and its injury potential determined, a normal nerve was transferred to a solution of known composition rich or deficient in potassium ions for a definite short time, taken out, allowed to drain and the end which had been crushed for the previous determination together with an additional 5 mm. cut off; the nerve was then re-mounted in the chamber and a fresh crush made. The action current was again tested and the injury potential measured: afterwards additional test stimuli were given at intervals if it was desired to follow the change in the action current response with time. When these observations were finished the nerve was put into aerated sea water to wash out the adhering salt solution. Subsequently it was remounted, the action current and injury potential tested as before, and if they had not been restored to values close to the original ones the washing and testing process was repeated a second or even a third time.

Results.—The action of potassium chloride rich solutions was twofold, affecting the injury potential and the action current simultaneously. When the potassium ion concentration was increased the action current decreased and then vanished, whilst the injury potential also decreased; with a further increase in the potassium ion concentration the injury potential also vanished. The potassium rich solutions exercised both of their effects fully in less than a minute (about 55 seconds) which was the minimum time required to immerse the nerve for 15 seconds, drain, mount it with all the necessary precautions and make a reading. Moreover, whether the nerve had been soaked in the potassium rich solution for 2 hours or merely for a few seconds the effects were completely reversible. The injury potential could be restored by vigorous agitation for about half a minute in sea water, but the action current was not always restored so quickly, especially if the nerve had been in the depressed state for some time, or in solutions containing large amounts of potassium: further washing was then necessary for complete recovery.

Conduction and the Action Current.

A nerve which had been immersed in sea water to which had been added isotonic potassium chloride solution, so that the total potassium ion concentration was about five times that in ordinary sea water or that in *Maia* blood, exhibited no action current on stimulation and the injury potential was depressed by about 15 millivolts. However, the minimum concentration of potassium ions did not seem to be very critical and differed somewhat with nerves from different crabs, hence the value quoted above is a mean obtained from about a dozen experiments in which the extreme values observed were 3·8 and 6·0 times the concentration of potassium present in ordinary sea water. This inexcitability was easily reversible. Provided that the nerves had not been in the depressed state for more than about an hour, washing for about 30 minutes restored completely the action current, no further increase—often a decrease—being observed with more prolonged washing.

The first series of experiments was done in May and a second series in September gave the same mean value for the potassium ion concentration required to abolish the action current, therefore it is unlikely that there was any marked seasonal variation in the susceptibility of nerves which had been soaked for an hour in sea water especially as they had been selected from animals which were neither about to moult nor had recently moulted. In the September experiments which were done to confirm the earlier ones, since it was desired only to know that no irreversible change had taken place the nerves were simply washed for an hour in aerated sea water and then tested.

Some experiments were also done to see whether the usual physiological antagonism between potassium and calcium could be demonstrated with crab's nerve. First, the effect of calcium enriched sea water was investigated. Even when a nerve had been immersed for 15 minutes in 90% of isotonic calcium chloride solution and 10% of sea water, so far as could be told by observations of the galvanometer deflections during the next hour, the action current was unaltered both in size and form. Then, in experiments performed with sea waters enriched with both potassium and calcium simultaneously it was found that those containing five times the usual amount of potassium and an equimolecular concentration of calcium did not produce inexcitability although they reduced the action current to less than one-half of its initial value. (To save space the results of these experiments are not tabulated; instead the results of some similar experiments with artificial sea waters enriched with potassium and calcium chlorides given in Table VI may be taken as representative.) The effect was the same whether the nerve was immersed in the solution for a minute or for 15 minutes, but with other solutions containing the same concentration of potassium and a smaller one of calcium the influence of the duration of immersion became more marked. An attempt was made to relate the reduction in the action current with the immersion time using certain fixed potassium calcium ratios but this was unsuccessful because in the same solution nerves from different crabs succumbed in very different times. The effect of calcium in fortifying the nerve against inexcitability was found to be reversible; if a nerve which had been treated with a calcium rich sea water, or a sea water containing both added potassium and calcium, was washed for a few minutes in sea water and then immersed in a potassium rich solution it succumbed just as quickly as one which had had no previous treatment other than soaking in sea water.

In order to test whether the dilution of the other major constituents of the sea water by the addition of the isotonic potassium chloride and calcium chloride solutions, and whether the presence of the minor constituents (Harvey, 1928; Clarke, 1924) had anything to do with the effects observed the experiments described above were repeated using artificial sea waters rich in potassium or calcium or in both of these ions together. The results were in close agreement with those obtained already, therefore they will not be discussed.

In a further experiment with an artificial sea water which was intended to be as free of potassium as possible, and which was found by analysis to contain less than 1 γ of potassium per cubic centimetre the action currents in response to 3 second tetani were slightly larger than normal. However, since the increase was only from 5 to 8% this observation requires further confirmation, but it seems of interest in showing that a nerve can give normal or nearly normal responses even when potassium is present in very small amounts in the surrounding medium.

Lest the potassium chloride treated nerves might have been more susceptible to the handling and re-crushing than normal nerves the results of the experiments so far described were checked and separated from the injury potential effect by the use of a different technique. The method was to mount an untreated nerve, crush its end in the usual way, and observe the action current responses to two or three test tetani applied at the pair of electrodes nearer to the galvanometer and spaced sufficiently to allow of complete recovery in the intervals between them. The responses to two or three test tetani applied at the electrodes at the central end were also observed. Then potassium chloride enriched sea water was dropped on to the middle third of the length of nerve which was suspended between the two sets of stimulating electrodes.

After a short interval another test tetanus was given at the central pair of electrodes and further tetani were given at intervals. Finally test tetani were given at the peripheral stimulating electrodes to see whether the nerve had deteriorated or the "crush sealing over" effect had become appreciable during the experiment. It was found that a solution containing five times the amount of potassium normally present in sea water produced a block between the two pairs of electrodes in less than a minute and there was no transmitted effect on the injury potential, hence the result by this method was in good agreement with that obtained by the total immersion method.

The Injury Potential.

When a nerve had been rendered inexcitable by immersion in sea water to which had been added isotonic potassium chloride solution so as to make the potassium concentration five times its normal value the injury potential was reduced by about 15 millivolts. Higher potassium concentrations reduced the potential still further: after immersion in sea water containing 13 times the normal potassium concentration the injury potential was 3–5 millivolts, and after immersion in sea water containing 17 to 26 times the normal potassium concentration the injury potential vanished completely. Again, as with the abolition of the action current the potassium concentration required to abolish the injury potential was not critical and somewhat different amounts were needed even for nerves which had had approximately equal injury potentials initially. Presumably these differences were due to differences in the amount of short circuiting taking place in the tissues.

Similar experiments with artificial sea waters rich in potassium chloride gave results in good agreement with those mentioned above, hence they will not be discussed further. Four other series of experiments with artificial sea waters are of interest. The first was with a sea water which was made up to contain no potassium. Nerves which had been immersed in this solution gave injury potentials which were in the neighbourhood of 40 millivolts instead of the usual 30 millivolts. When they had been washed for less than 3 minutes in ordinary sea water they again exhibited normal injury potentials, Table V. The second and third series of experiments were done with artificial sea waters in which the potassium concentration was maintained constant at the normal value, whilst in one the calcium and in the other the magnesium concentration was varied. Neither calcium nor magnesium was found to have any effect in an hour on the injury potential within the experimental error of determination. The fourth series was made with several different concentrations of potassium and calcium because it was thought possible that since the calcium was capable of preventing the onset of reversible inexcitability it might do so by preventing the potassium from depressing the injury potential. However, the results were negative, a depression of the injury potential being observed although the action current was not abolished, Table VI.

Table V.—The Effect of Potassium Deficient Artificial Sea Water on the Injury Potential.

Date.	Injury potential of nerve after an hour in aerated sea water, mv.	Duration of immersion in K deficient sea water, mins.	Injury potential after immersion, mv.	Injury potential after washing 2–3 minutes in normal sea water, mv.
21.6.32	31·7	13	38·0	32·1
21.6.32	33·7	5	42·4	32·1
22.6.32	27·2	6	36·4	28·0
22.6.32	22·2	20	27·0	23·3

Table VI.—The Effects of Artificial Sea Waters Enriched with Potassium and Calcium Chlorides.

Date.	Normal nerve after an hour's soaking in aerated sea water.		Nerve after immersion in $K^{\cdot}$ and $Ca^{\cdot\cdot}$ enriched artificial sea water.			
	Injury potential, mv.	Action current deflection, mm.	A*.	Duration of immersion, mins.	Injury potential, mv.	Action current deflection, mm.
9.6.32	19·4	15	$3K^{\cdot} + 5Ca^{\cdot\cdot}$	121	5·5	3
1.9.32	25·7	32	$4{\cdot}8K^{\cdot} + 5{\cdot}8Ca^{\cdot\cdot}$	11	18·8	3
7.9.32	28·6	35	$4{\cdot}8K^{\cdot} + 5{\cdot}8Ca^{\cdot\cdot}$	5	12·0	15
	23·6	38	$4{\cdot}8K^{\cdot} + 5{\cdot}8Ca^{\cdot\cdot}$	6·5	18·6	13
8.9.32	35·9	49	$4{\cdot}8K^{\cdot} + 5{\cdot}8Ca^{\cdot\cdot}$	2	21·7	22
10.9.32	35·5	24	$4{\cdot}8K^{\cdot} + 5{\cdot}8Ca^{\cdot\cdot}$	15	21·7	4
12.9.32	27·2	44	$4{\cdot}8K^{\cdot} + 5Ca^{\cdot\cdot}$	15	16·0	7
13.9.32	28·4	20	$4{\cdot}8K^{\cdot} + 5Ca^{\cdot\cdot}$	5	19·1	15

A*—Potassium and calcium concentrations given as multiples of the usual concentrations in Plymouth sea water.

An attempt was made to show in *Maia* nerve, as Macdonald (1902) showed in medullated nerve, a linear relation between the logarithm of the potassium concentration in the external medium and the injury potential, but it was not very successful because the reduced injury potentials obtained after treatment with potassium chloride rich solutions were relatively less reproducible than the larger ones of untreated nerve. A possible reason is that as the sodium chloride content of the solutions was decreased and the potassium chloride content was increased the electrical conductivity also increased and the short circuiting due to fluid retained between the fibres would become more important as the injury potential diminished. However, from the results given it seems that with crab's nerve there is no very serious departure from the logarithmic relation, fig. 2, over the concentration range of 1-30 times the amount of potassium present in normal sea water. In a few experiments with nerves which had been immersed in a solution containing 80% of isotonic potassium chloride and 20% of sea water the "injury potential" remained zero, and potentials in the opposite sense were not obtained.

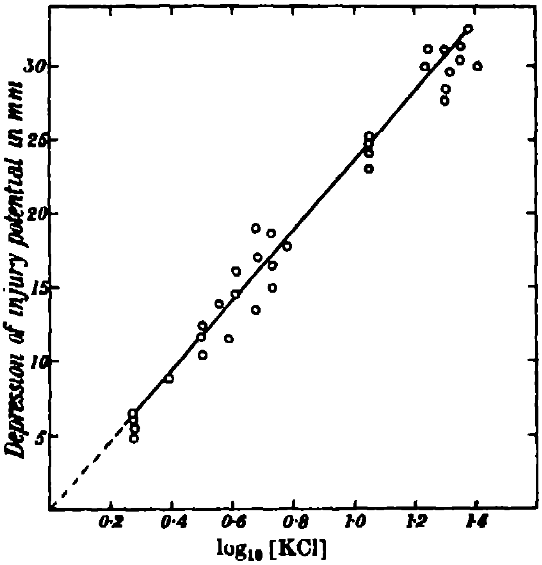


FIG. 2.—The relation between the potassium chloride concentration in the solution surrounding the nerve and the injury potential. The potassium concentration occurring in sea water (0·0388 gm. per 100 gm.) has been taken as unity and the concentrations in the other solutions have been expressed as multiples of this.

Some experiments were performed with sea water made hypertonic with added sodium chloride, of "analytical reagent" quality, in order to partly dehydrate the nerve and thus increase the effective potassium concentration within the fibres. Preliminary experiments showed *Maia* nerve very sensitive to osmotic changes and that immersion in sea water which had had its osmotic pressure increased by about 25% produced the maximum dehydration that it would stand without loss of the action current. A nerve which had been so treated gave an injury potential which was 5–10% higher than that which it had given previously after immersion in ordinary sea water. After a second washing in ordinary sea water the injury potential fell to approximately its former value, Table VII.

Table VII.—The Effect of Dehydration by Hypertonic Sea Water on the Injury Potential.

Date.	Injury potential of nerve after an hour in aerated sea water, mv.	Duration of immersion in hypertonic sea water, mins.	Injury potential after immersion, mv.	Injury potential after half an hour's washing in aerated sea water, mv.
23.6.32	27·8	5	30·4	25·9
25.6.32	36·9	5	40·0	37·2
	19·7	4	21·6	20·4
	27·4	5	28·8	26·8

It must be emphasized that all the experiments described above apply only to nerves in a completely resting condition. The two other factors affecting the injury potential—stimulation and asphyxia—are discussed in later sections of this paper.

The Reversible Inexcitability of Freshly Dissected Nerve.

The action of solutions rich in potassium ions is of interest in connection with Furusawa's (1929) observation of the reversible inexcitability which occurs in freshly dissected nerve. Often he found that *Maia* nerves when tested immediately after preparation by his "pulling out" method gave no action currents. Washing for a few minutes in sea water removed the inexcitability and prohibited its reappearance. At the beginning of the present work these facts were confirmed and it was noted that this inexcitability usually appeared in nerves prepared by Furusawa's method. On the other hand, it occurred in about 50% of the nerves prepared by Levin's method, whilst the other 50% gave small action currents which gradually disappeared, but could be brought back and increased by washing with sea water. The work of Dulière and Horton (1929) and of Horton (1930) on the inexcitability produced by potassium ions in frog muscle suggested that potassium might be responsible here also, and led to the adoption of the routine of suspending the nerve in a large volume of aerated sea water for about an hour. This time was found to be sufficient for complete recovery from the effects of dissection, and inexcitability was not observed to return within the time required for an experiment.

The fact that Furusawa's method was found more prone to yield inexcitable nerves suggested that the phenomenon might be associated with damage to the fibres at the surface of the trunk. It was soon found that potassium salts did leak out, or were present on the surfaces of nerve trunks, even when they had been prepared by Levin's method, for if a small volume of sea water, protected against evaporation, was used for washing a number of such nerves in succession it became less and less effective in restoring excitability and finally failed to do so. Initially 2 c.c. of sea water were taken and by the time that 15–20 nerves had been washed in it for 5 minutes each the volume had decreased to nearly one-half, as no attempt was made to remove all the surplus fluid adhering to the nerves for fear of injuring them, and the solution had lost its power of restoring excitability or it would render a normally excitable nerve inexcitable. Chemical analysis of a 1 c.c. sample of this residual solution, by the method described in the section on *Maia* blood and nerve, showed that the potassium concentration had increased fivefold (two results). As a control operation a similar number of nerves was given a preliminary soaking in a large volume of aerated sea water and then immersed for 5 minutes in a sample of sea water which occupied a volume of 2 c.c. initially. At the end of the process this sea water would restore the action current in a freshly dissected nerve and the potassium content had increased by less than 10%.

The above experiments considered in conjunction with those on the reversible inexcitability produced by potassium rich solutions suggest strongly that the inexcitability of freshly dissected nerve is due to the presence of potassium salts between the fibres. Probably most of the potassium comes from small unavoidable injuries inflicted on the nerve or on the adjacent muscle fibres during the dissection, although as is shown later some potassium salts may leak out from the nerve as a result of stimulation which may cause such a thing to happen if carried to the point of fatigue. But whatever the cause the leakage does not continue for very long, because as shown above the amount of potassium which escaped from 15–20 nerves which had been soaked for an hour previously in aerated sea water was very small (see also the section on potassium leakage); moreover, once excitability had been restored by thorough washing it was retained for at least 3 hours: in fact, nearly always it returned after a nerve had been asphyxiated and allowed to recover.

Furusawa (1929) also noted that during the spring and again in August and September nerves were more liable to exhibit inexcitability than at other seasons. In the experiments described here care was taken not to use crabs which had obviously just moulted and no vernal increase in susceptibility was observed nor was there any change in the potassium content as evinced by the injury potential; but during the first week in October, owing to a temporary shortage of material, a few specimens of *Maia* which seemed to have moulted that season were used. Although they gave normal injury potentials nerves from these crabs seemed to become inexcitable more readily than usual, but no special observations were made to test this impression. If Furusawa sometimes employed nerves from recently moulted crabs then this increased susceptibility may be explained on the basis of the following observations. Paul and Sharpe (1916) noted that in *Cancer pagurus* just before the moult there was a decrease in the blood volume and after the moult this was followed by an increase which might be as much as tenfold in the next 10 days. They found that during the whole process the blood calcium remained constant at about 60 mg. per 100 c.c. and that the calcium which had been present in the hepato-pancreas before the moult disappeared. They suggested that the hepato-pancreas serves as a storage organ and that calcium is taken up or liberated as required. Pantin (1931, *a* and *b*; Pantin and Weil, 1931) in his studies on *Gunda ulvæ* has shown that this worm can withstand large changes in the salinity of its environment and can regulate its osmotic pressure to some extent only provided that sufficient calcium is present. From the experiments of Hukuda (1932) it may be gathered that *Cancer pagurus* has some power to regulate its osmotic pressure; this may be connected with the constant level of blood calcium which it exhibits. As Margaria (1931) has shown, *Maia* is very different; this animal has no power to regulate its osmotic pressure and simply comes into equilibrium with its environment. If we bear in mind also the experiments on the inexcitability produced in *Maia* nerve by potassium rich solutions and its prevention by calcium ions it seems possible that in *Maia* blood there is some disturbance in the potassium calcium ratio at the time of moulting—the liver may fail to supply sufficient calcium just after the moult.

The Effects of Rubidium, Cæsium, Lithium, and Ammonium on the Action Current and Injury Potential.

Since rubidium and cæsium ions resemble potassium ions in many properties and all three ions have nearly the same mobility in aqueous solution it was thought interesting to try their actions on *Maia* nerve; also the action of lithium ions.

Sea water enriched by the addition of isotonic rubidium chloride solution rendered a nerve inexcitable in less than a minute when the molar concentration of rubidium chloride was eight to nine times the molar concentration of potassium in sea water. This inexcitability was found to be reversible, like that produced by potassium, and the nerve could be restored completely by washing in aerated sea water.

Sea water enriched with cæsium chloride solution also produced reversible inexcitability, but a molar concentration of about 13 times the molar concentration of potassium in sea water was required.

In nerves rendered inexcitable by rubidium or cæsium chloride nearly always after 15–20 minutes there was some depression of the injury potential, but since it was about 4 millivolts, an amount not much greater than the differences between successive determinations of the whole potential, it was impossible to measure it accurately, Table VIII.

Table VIII.

Date.	Nerve after an hour in aerated sea water.		Nerve after 3 minutes in sea water with added rubidium chloride (0·09 N).		Nerve after recovery in aerated sea water.	
	Injury potential, mv.	Action current deflection, mm.	Injury potential, mv.	Action current deflection, mm.	Injury potential, mv.	Action current deflection, mm.
8.8.32	30·4	25	28·3	0	29·5	19
	33·2	34	29·8	3	31·8	30
9.8.32	20·5	15	14·8	0	17·0	10·5
			Sea water with added cæsium chloride (0·13 N).			
9.8.32	28·4	30	24·2	3	29·0	25
10.8.32	29·3	27	29·1	1	28·4	25
	31·7	42	27·6	5	28·7	34

To eliminate the effects of potassium, experiments were performed with artificial sea waters which were free from potassium but contained varying amounts of either rubidium or cæsium chloride. It was found that 2·0 gm. molecules of rubidium chloride or 3·2 gm. molecules of cæsium chloride were as effective as 1 gm. molecule of potassium chloride in abolishing the action current. The increase in the injury potential due to the potassium deficiency was greater than the depression caused by either rubidium or cæsium ions.

These experiments and the earlier ones with potassium indicate that, within certain limits the injury potential of a nerve could be given an arbitrary value by immersion in a solution containing a suitable concentration of potassium chloride, and then the action current could be reduced as much as desired by the addition of rubidium chloride or cæsium chloride.

In some further experiments the effect of increasing the calcium chloride concentration in a rubidium chloride or cæsium chloride sea water which would just abolish the action current was investigated. It was found that a calcium chloride concentration giving five times the usual calcium concentration in sea water would prevent in a fair degree the reversible inexcitability which would otherwise have been produced by the rubidium or cæsium ions, Table IX.

Table IX.—The Action Current deflections given by Nerves.

Date.	After an hour in aerated sea water, mm.	After 3 minutes in a potassium deficient artificial sea water $0 \cdot 085$ N in RbCl and $0 \cdot 050$ M in $CaCl_2$, mm.	After washing in normal sea water and 3 minutes' immersion in artificial sea water $0 \cdot 085$ N in RbCl, mm.
9.12.32	52	39	2
	17	15	0
	37	30	4
		After immersion in a similar artificial sea water but $0 \cdot 114$ N in RbCl and $0 \cdot 05$ M in $CaCl_2$, mm.	After washing in normal sea water and 3 minutes' immersion in artificial sea water $0 \cdot 114$ N in RbCl, mm.
10.12.32	59	48	7
	65	53	5
	36	34	2
	54	40	2

The action of ammonium chloride was also tested. A concentration equivalent to five times the amount of potassium present in sea water usually produced inexcitability, but in two experiments out of six the effect was not reversible. Larger concentrations of ammonium chloride damaged the nerve irreversibly, abolishing both the action current and the injury potential. These toxic effects of ammonium ions are not very surprising, since at p_H $8 \cdot 1$ they could hardly be expected to behave exactly like alkali metal ions.

Potassium deficient artificial sea waters containing lithium chloride in concentrations up to $0 \cdot 19$ molar had no effect on the action current response or the injury potential as determined by observations made over a period of about an hour.

The Leakage of Potassium from Stimulated Nerve.

Experimental.—Nerves were stimulated in a small volume of sea water and samples withdrawn in order to determine by chemical analysis the rate at which potassium salts escaped from the tissue: at the same time control experiments were made (*a*) with unstimulated nerves immersed in sea water, and (*b*) on the rate (if any) at which evaporation caused the potassium concentration to increase in a sample of sea water without any nerves in it, but under the same conditions. This last experiment was done so as to enable the measurements to be expressed as absolute rates of leakage of potassium salts from stimulated and resting nerve.

To avoid the inexcitability due to local fatigue it was thought better to have the stimulating electrodes situated at opposite ends of the nerve and to pass the stimulating current between them; also they were so arranged that the ends of the nerve made contact with them above the liquid surface, thus obviating leakage of the stimulating current through the sea water.

For these experiments a nerve chamber was made from a block of paraffin wax approximately 5 inches by 3 inches by 1 inch, to the design shown in fig. 3. Three grooves each 100 mm. long, 10 mm. wide, and 9 mm. deep were cut parallel to the longer side. Through the wax walls into each of the outer grooves (A and C) were led two silver wires, one of which was placed about 3 mm. from one end wall (1), and 7 mm. from the floor; the other was placed 4–5 mm. from the opposite end wall and 7 mm. from the floor (2). A glass rod was fixed transversely across the three grooves and above the (2) electrode.

Four or five pairs of nerves were selected, each pair being taken from a different crab, dissected as quickly as possible, and suspended in a large volume of aerated sea water. The preparation took nearly an hour and the nerves were allowed to rest in the sea water until the one last dissected had been soaking for half an hour; during this time they were all cut to the same length, about 11 cm., and where necessary fresh cottons were tied to the ends. Then a little sea water was placed in each of the outer grooves (A and C) of the chamber, one set of four or five nerves was taken and mounted in one of the grooves. The nerves were so arranged that they lay side by side on the floor, their ends were passed beneath the silver wire electrodes without crossing, and they were secured in position by embedding the attached cottons in plasticene placed on the end wall near the (1) electrode and on the glass rod above the

(2) electrode, fig. 3. Similarly the companion nerves were mounted in the unoccupied outer groove. A glass cover was then sealed on so as to close the chamber completely except for a small strip about 4 mm. wide lying beyond the electrodes and the glass rod. The purpose of the cover was to reduce the evaporation correction as much as possible, the openings being left for the insertion of the tips of pipettes for the withdrawal of samples.

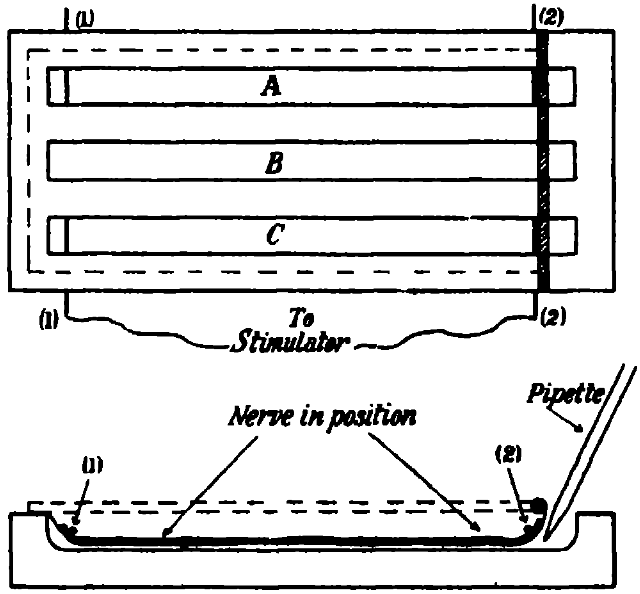


FIG. 3.—The nerve chamber used in the stimulation experiments. A, B, C, grooves for nerves; (1), (2), silver wires. The dotted lines indicate the position of the glass cover.

The chamber was rocked gently in a direction parallel to its longer side about once in 3 seconds for approximately 10 minutes, then it was tilted so that the end bearing the glass rod became the lower and the sea water in which the nerves had been immersed was drained off and removed as completely as possible with a pipette. Into each of the grooves was pipetted 5 c.c. of fresh sea water and the rocking was re-started; also, two 1 c.c. samples of the sea water were taken from the main supply. At the end of 5 minutes the rocking was stopped and a 1 c.c. sample was taken from each groove. The rocking was re-started and tetanisation of one set of nerves was commenced. A Harvard coil was used with the secondary at 8·5, the frequency having been adjusted to approximately the required value by loading the hammer of the interruptor with plasticene. At the end of 5 minutes the rocking was stopped whilst a 1 c.c. sample was taken from each groove. The rocking was restarted and tetanization was continued for another 5 minutes then both were stopped and three more 1 c.c. samples were taken. Again, the rocking was re-started and at the end of 5 minutes a final batch of three 1 c.c. samples was taken. The chamber was opened, the lengths of stimulated and unstimulated nerve which had been immersed in the sea water were cut out, blotted on " gravimetric " filter paper, put into weighing bottles and weighed.

The potassium determinations were carried out on the fluid samples in the manner described for 1 c.c. samples of *Maia* blood in an earlier section of this paper, but as the experiments were done before the syringe pipettes were obtained ordinary glass pipettes of N.P.L., B standard were used.

Results.—In nearly every experiment the evaporation was reduced to such an extent that in the 20 minutes in which the samples were taken no measurable change in the potassium concentration had occurred. A more accurate estimate was obtained in two experiments by omitting to take the sample at the end of the first 5 minutes, the rocking being continued and a sample taken after 45 minutes had elapsed. In these the increase in the potassium concentration was about 2%, hence in the 20 minutes in which the samples were usually taken the evaporation correction was only 0·9% of the potassium concentration, a quantity not very much greater than the experimental consistency with which the potassium concentrations in the samples could be compared. The increase in the potassium concentration in the solution surrounding resting nerves was small and consequently it was impossible to measure the rate of potassium leakage with any accuracy, but usually it was between 0·001 and 0·002 mg. potassium per gram of nerve per minute, Table X. The rate of leakage of potassium from nerve stimulated by 20 make and break shocks per second was quite large—in the last 1 c.c. sample of sea water taken, often the potassium concentration had increased by nearly 50% above its initial value. In Table X it will be seen that the amount of potassium leaking out from a gram of nerve was greater in the second 5 minutes of stimulation than in the first 5 minutes, but that after cessation of stimulation the rate of leakage was less than it had been during the periods of stimulation, although it remained four to five times as great as in resting nerve.

With a frequency of about 100 make and break shocks per second the rate of potassium leakage during the first 5 minutes was as great as it was during the second 5 minutes, and the rate of leakage in the 5 minutes following stimulation was about the same as in the experiments at the lower frequency, Table X.

With a still higher frequency of about 140 make and break shocks per second the maximum rate of potassium leakage was attained during the first 5 minutes of stimulation, it fell off in the succeeding 5 minutes, and in the 5 minutes after the stimulation it was rather smaller than in the experiments at the lower frequencies, Table X.

Table X.—The Leakage of Potassium Salts from Resting and Stimulated Nerves.

Experiment.	Potassium leakage in milligrams × minutes per gram of nerve.		Approximate frequency of stimulation, per sec.
	Resting nerve.	Stimulated nerve.	
2	0·0075	A 0·133 B 0·142 C 0·048	40
3	0·008	A 0·139 B 0·145 C 0·064	40
4	0·006	A 0·131 B 0·154 C 0·104	40
7	0·010	A 0·056 B 0·114 C 0·042	40
8	0·009	A 0·158 B 0·154 C 0·094	100
9	0·0115	A 0·147 B 0·151 C 0·107	100
10	0·0055	A 0·173 B 0·159 C 0·044	140
11	0·008	A 0·179 B 0·148 C 0·034	140

A, during the first 5 minutes of stimulation.
B, during the second 5 minutes of stimulation.
C, during the first 5 minutes following stimulation.
The potassium leakage from resting nerve in 5 minutes is a mean of three determinations.

As a corollary to the results just described and to the earlier ones on the effect of potassium on the injury potential it seemed possible that the application of a drop of potassium chloride rich solution would set up an impulse in a nerve since it would be equivalent to the breaking of a constant current, and conversely that the application of a drop of potassium deficient solution would be equivalent to the making of a constant current.

Some experiments were done with nerve-muscle preparations from the claws and walking legs of *Maia*. A drop of isotonic potassium chloride solution caused a single and occasionally a double twitch of the muscle within a few seconds of application to the nerve and immediately afterwards a test stimulus at the central end indicated a block. In none of the experiments in which potassium free artificial sea water was applied to the nerve was a twitch observed. The probable explanation of the ineffectiveness of the potassium deficient solutions is that they were not sufficiently free from potassium ions to set up such a large e.m.f. as did the isotonic potassium chloride solution.

The Effects of Asphyxia.

Cowan and Feng have repeated Furusawa's (1929) experiments on the rate of disappearance of the " retention " of action potential in nerves in oxygen and in nitrogen. Their results which confirm those of Furusawa have been described already (Beresina and Feng, 1933).

Furusawa's (1929) work showing that the injury potential is maintained in the presence of oxygen and fails in its absence has also been confirmed. However, the main reason for doing experiments with asphyxiated *Maia* nerve was, that since it contains so much potassium the diminution in the injury potential might have been accompanied by a leakage of potassium salts. There is little doubt that if asphyxiation were carried to such a length as to inflict permanent damage on a nerve potassium salts would leak out and could be determined by a chemical method, but such a result would have been of little interest and hence the more delicate experiments following were done in the hope that they would have some physiological meaning.

A nerve was rendered inexcitable by immersion in sea water containing only just sufficient added potassium chloride, drained of surplus fluid and mounted. After the absence of an action current had been established, a stream of moist oxygen through the chamber was started and the nerve was given two 1 second test stimuli each half-hour. The experiment was continued for several hours; occasionally the chamber was opened and a fresh crush was made to eliminate the " crush sealing over " effect. At the end of such an experiment it was found possible to restore the action current by washing the nerve in aerated sea water. In one instance a nerve remained inexcitable for 40 hours in moist oxygen and at the end of that time washing in sea water restored what action current there remained, about 5% of the initial value.

From the experiments just described it seems that a nerve which has been rendered inexcitable by a minimal concentration of potassium does not recover in oxygen alone; washing is always necessary; moreover, such experiments can be regarded as controls for some asphyxiation experiments described below.

When a nerve had been asphyxiated until the action current had vanished, and the asphyxiation continued for a further period of about half an hour, the subsequent admission of oxygen brought about a restoration of both the action current and the injury potential. Since oxygen alone was incapable of removing the inexcitability, caused by excess of potassium salts between the fibres of a nerve trunk, the restoration of the action current, after a short period of complete asphyxia, would indicate that sufficient potassium does not leak out from a nerve during one of these experiments to produce the inexcitability.

In all the experiments recorded here asphyxiation proceeded more rapidly than in Furusawa's (1929) experiments. Probably this was because the present ones were done at a higher temperature, and because, at the beginning of a period of anoxia, the nerve chamber was washed out with a fast stream of nitrogen.

In three experiments in which asphyxiation was continued for an hour before oxygen was admitted, even after making a fresh crush, both the injury potential and the action current remained depressed. The injury potential was reduced to about 70% of its initial value while the action current was reduced to about 10%. Washing in aerated sea water restored the injury potential to about 80% of its initial value, and the action current to between 50 and 60% of its initial value. These experiments suggest that the sea water restores the nerves by washing away some toxic substance. This may well be potassium salt since it has already been shown that none of the other cations of sea water influences appreciably the galvanometer deflection due to the action current, and that a nerve rendered inexcitable by potassium cannot recover in oxygen. It is emphasized, however, that washing is only necessary to restore nerves which have been asphyxiated to the point of slight permanent injury, and that the effect is a secondary one.

Discussion.

From the results of the chemical analyses of *Maia* blood and nerve it can be calculated that the blood and the nerve plasma must be very nearly if not exactly isotonic with one another and with sea water. Indeed it would be very difficult to imagine things otherwise when one recalls the fragility of these nerves and their sensitivity to changes in osmotic pressure. It follows, then, that in these nerves the potassium and other ions must be in solution since any adsorption would render the nerve plasma hypotonic to the blood. Hence there is no possibility that local accumulations of potassium salts exist " on or in the surfaces of the axons " of the fibres or " also on or in their dendrites " as Macallum (1932) claims to have shown in medullated nerve.

The reversible inexcitability which is exhibited when potassium rich tissues are immersed in a potassium rich solution is probably a far more widespread phenomenon than is at present realized. In frog muscle it has been investigated by Dulière and Horton (1929) and by Horton (1930); in the plant cells of *Nitella clavata* by Osterhout and his collaborators (1931); recently Feng (1933) has shown that it occurs in the sensory end organs of frog's skin. It has long been known that potassium salt solutions will depress and block medullated nerves (Menten, 1912; for earlier references see Höber and Strohe, 1929), but owing to difficulties in technique the minimum concentration required to produce inexcitability and the recovery process do not seem to have been investigated. However, some observations by Forbes and Ray (1923) are very suggestive of the influence of potassium: using cats they found that, of freshly dissected nerve trunks 14% were inexcitable when tested immediately. Also, a nerve cut off from its blood supply and left lying in a wound completely surrounded by dissected tissues rapidly became inexcitable. Such a nerve could be restored by washing in Ringer's solution—the relatively rapid restoration certainly suggests an ionic process.

The ratio of the minimum ionic concentrations of potassium, rubidium, and cæsium required to produce reversible inexcitability is in the ratio 1 : 2·0 : 3·2, whilst the ratio of their atomic weights is 1 : 2·2 : 3·4. This relationship suggests that the action of these ions is a simple physical one, and possibly that the initial stages of propagation of a nerve impulse are due to a physical change rather than a chemical one (*cf.* Hill, 1932, *b*).

In these experiments neither rubidium nor cæsium was observed to depress the injury potential appreciably, but with frog's medullated nerve Netter (1927), Höber and Strohe (1929) found that, after producing a temporary rise, rubidium depressed the injury potential considerably, whilst cæsium had no effect. It is possible that greater effects of rubidium and cæsium ions on the injury potential would have been observed with crab's nerve had the time between immersion in the solution and the determination of the injury potential been longer. Again, in these experiments when the inexcitability which would ordinarily be produced by potassium ions was prevented by the addition of calcium chloride, the depression of the injury potential was still manifested, but with frog's nerve Höber and Strohe (1929) found that calcium salts would prevent the depression of the injury potential due to added potassium salt.

The speed with which the injury potential responds to changes in the potassium ion concentration in the surrounding medium and the perfect reversibility of the changes adds to the evidence that the action of potassium is ionic and that the nerve membrane is permeable to potassium ions. The fact that other cations alter the injury potential little shows that the membrane of resting nerve is practically impermeable to them. Furthermore, when the results of the analyses of *Maia* blood and nerve, the sense of the injury potential and the direction in which it moves when potassium rich solutions are applied are considered in the light of Beutner's work (1920, 1933) it follows that the non-aqueous membrane bounding the nerve must have acidic properties.

The results of the chemical analyses indicate that the potassium concentration in *Maia* nerve is about 13 times as great as in the blood. The maximum possible injury potential which this concentration difference could set up at

$$17^\circ \text{ C. is} = \frac{RT}{nF} \log_e \frac{\text{C inside}}{\text{C outside}} = 0\cdot058 \log_{10} 13 = 64\cdot6 \text{ mv.}$$

From the measurements of the injury potentials of resting nerves and the later ones on the effects of different potassium concentrations on the injury potential, it is clear that under the conditions of these experiments less than one-half of the maximum e.m.f. was obtained. The loss might be due to short circuiting in the tissues.

It will be noted that although e.m.f. measurements are considered here and in other parts of this paper no attempt has been made to introduce ionic activities rather than concentrations. The reason is that all the solutions inside and outside the nerve must have had very nearly the same "ionic strength" (Lewis and Randall, 1923, chap. 28) and in order to express results in terms of activities the potassium ion concentrations inside and out would merely have to be multiplied by factors which, within the experimental error of ± 5%, would probably have the same value.

From the results of the chemical analyses it would be expected that a potassium concentration of 13 times the normal in the solution surrounding the nerve would abolish the injury potential, but in the experiments with potassium chloride enriched sea waters an e.m.f. of 3-5 millivolts remained and a concentration of 17–26 times the normal was required to make the injury potential zero. There are three possible explanations of this difference: as has already been suggested, some inactive tissue or sea water was included in the nerve samples analysed; the 3–5 millivolts is due to an asymmetry, in the nerve membrane, of the type described by Cremer; some of the potassium salt in the external solution becomes involved in a Donnan equilibrium across the nerve membrane. At present it is impossible to distinguish between these possibilities, but probably the first and third are the important ones. Although the accumulation of potassium within living nerve fibres could not be explained by a Donnan effect (*cf.* Osterhout, 1931, on *Valonia*), there seems to be no reason why Donnan distributions should not affect the potentials given by nerves treated with potassium rich solutions. If we assume that the volume of solution surrounding the nerve is infinite compared with the volume of the nerve plasma, then the Donnan expression for the membrane potential due to the asymmetric distribution of potassium ions reduces to the form

$$E = \text{const.} \times \log \frac{C_1 + \sqrt{C_1^2 + 4C_2^2}}{2C_2}$$

where C_1 is the concentration of potassium ions ordinarily present in crab's nerve before treatment, and C_2 is the potassium ion concentration in the solution with which the nerve is treated. Now if C_2 is small compared with C_1, the expression approximates to $E = \text{const.} \log C_1/C_2$, giving a nearly linear relation between the depression of the injury potential and the logarithm of the potassium ion concentration in the outside solution; but if C_2 is not small compared with C_1, then the relation is no longer a linear one; instead, as the potassium ion concentration in the surrounding medium is increased, the depression of the injury potential is less manifest, and finally it reaches a fixed value. The line plotted in fig. 2 would bend over towards the x-axis, and become asymptotic to a fixed potential. Thus "reversed injury potentials" would never be attained even when the solution surrounding the nerve contained only potassium salt.

When *Maia* nerves were stimulated in a small volume of sea water there was a definite loss of potassium salts and the loss continued at a slower rate after stimulation had ceased. It is a little difficult to decide what the results mean. The effect of the higher frequencies in causing greater potassium leakage may have been due to the greater number of stimuli administered, or it may have been due to an earlier onset of fatigue, as there is no doubt that although coil stimulation was used, and the earlier stimuli were barely maximal, the nerves were stimulated to a considerable degree of fatigue (Furusawa, 1929). The falling off in the leakage rate at the higher frequencies was probably due to some of the fibres becoming inexcitable with fatigue. The same difficulty of deciding how much of the potassium leakage is due to the passage of impulses and how much is due to fatigue has caused some controversy for frog muscle (Mitchell and Wilson, 1921; Wojtczak, 1927; Ernst and Scheffer, 1928). For plant cells of *Nitella* the results of Sen (1931) give a more definite answer. He found that the electrical resistance of a small volume of water surrounding a single cell decreased by about 20% when induction shocks, just sufficient to stop streaming within the cell, were administered; on the other hand, lethal shocks caused the resistance of the surrounding water to decrease by 300%. However, he did not identify the ions escaping.

The leakage of potassium from the nerve into the surrounding solution involves a loss of osmotic work or energy and it is of interest to evaluate this free energy decrease in order to compare it with the energy expenditures which have been measured as heat production.

For the purpose of calculating the osmotic work it is assumed that the true rate of potassium ion leakage was 0·0280 mg. per minute per gram of nerve at a stimulation frequency of 40 per second. Moreover, since potassium ions could not leak out unaccompanied, for simplicity, the further assumption is made that the companion anions (yet unidentified) were univalent. Then, the total ionic leakage becomes $5 \cdot 84 \times 10^{-10}$ gram equivalents per single impulse per gram of nerve at a frequency of 40 per second. Using the usual notation (see Lewis and Randall, 1923) the osmotic work is

$$- \Delta F = NE\phi = NRT \log \frac{C_1}{C_2}.$$

In this calculation the form

$$- \Delta F = 2 \cdot 303 \text{ NRT} \log_{10} \frac{[K^{\cdot}] \text{ inside nerve}}{[K^{\cdot}] \text{ outside nerve}}$$

will be used, and the potassium concentration ratio of 13—approximately that found by chemical analysis—will be used rather than any expression involving ionic activities. The osmotic work at 17° C. is

$$\begin{aligned} - \Delta F &= 2 \cdot 303 \times 5 \cdot 84 \times 10^{-10} \times 1 \cdot 989 \times 290 \cdot 1 \times \log_{10} 13 \text{ cal.} \\ &= 0 \cdot 864 \times 10^{-6} \text{ cal.} \\ &= 36 \cdot 12 \text{ ergs.} \end{aligned}$$

The most recent measurements of the heat production of *Maia* nerve are those of Beresina and Feng (1933). They found that the initial heat per single isolated impulse was $0 \cdot 73 \times 10^{-6}$ calorie per gram of nerve at 16·5° C. With the aid of their curve showing the effect of frequency on the rate of heat production, and taking the ratio of the initial heat to the recovery heat as 0·02, the recovery heat per single impulse can be calculated to be $38 \cdot 1 \times 10^{-6}$ cal. per gram at a frequency of 40 per second.

The fact that the energy loss, due to potassium leakage, and the initial heat are of the same order of magnitude is no more than a coincidence, since the former is a free energy change involving no liberation of heat, and the latter is a thermal change; moreover, it is unlikely that they have the same distribution in time. Hill (1932, *b*) has suggested that after activity the nerve is "re-charged" for further activity by a secretory process, whereby ions are restored to their former positions. Recently Feng and Hill (1933) have brought forward some evidence, which at least shows that in frog's medullated nerve the initial heat and the recovery heat do not arise from consecutive chemical reactions, in which the products of the first are the reactants in the second. If the potassium ions which have leaked out from crab's nerve are restored by a secretory process, then it is possible that the long continued recovery heat (Hill, 1929) represents the energy expenditure of this restoration. If this is true, then it follows that *Maia* nerves have a thermodynamical efficiency of about 2·3%—an efficiency of the same order as that with which the human kidney performs its osmotic work (Borsook and Winegarden, 1931).

The effects of asphyxia on nerve are manifested in two stages: in the first, recovery can be brought about by oxygen alone, but in the second it seems probable that potassium salts leak from the fibres. With medullated nerve many conflicting results have been reported as to the necessity of washing for complete restoration after asphyxia. An explanation of these differences would be that some workers carried asphyxiation to the point of producing potassium salt leakage and others did not.

Horton (1930) in his work on the inexcitability of frog muscle showed that treatment with Ringer's solution enriched with potassium chloride caused an increase in the excitation time, and Lucas (1908) showed that treatment with solutions enriched with calcium ions diminished the excitation time. On the basis of his electrical model of nerve Hill (1932, *b*) has suggested that potassium and calcium ions might act by changing the electrical resistance of the sheath and, consequently, the value of Rushton's (1927) "analytical unit." There are two possible mechanisms whereby this resistance change could be effected and they have some points in common.

(*a*) The current may be carried in the nerve sheath by ions and the conductivity may depend upon the mobilities of the ions in the material forming the sheath. If so, then it would be expected that the mobilities in that material of the three ions potassium, rubidium, and cæsium would be in the same order $1 : \frac{1}{2} : \frac{1}{3 \cdot 2}$, as their effectiveness in producing inexcitability.

There is no doubt that the nerve membrane is permeable to potassium ions, and a possible reason why they do not ordinarily diffuse out is that no anions diffusible in the membrane material are available in resting nerve. But when the nerve is excited, leaving out any possibility of a temporary rupture of the membrane, new ions, *e.g.*, lactate, may be formed and they may diffuse out accompanied by potassium.

Beutner (1933) has shown that in his artificial systems the addition of small amounts of narcotics or alkaloids to the water immiscible phase reduces greatly the observed potential difference. In nerve it is inconceivable that asphyxia could affect seriously the "activities" of the potassium ions in the aqueous phases, but it is quite possible that a metabolite or metabolites accumulating during asphyxia could pass into the membrane material, alter its properties and reduce the observed injury potential. Beutner and Lozner (1930) have given experimental evidence that non-oxidized materials, which yield acids insoluble in water—for example, heptylic alcohol or aldehyde—are electrically negative relative to their oxidation products. The oxidative removal of such a metabolite would result in a restoration of the injury potential.

(*b*) An alternative explanation in terms of emulsions involves changes in permeability. If the nerve is bounded by a water in oil emulsion, then potassium, rubidium, and cæsium ions could produce inexcitability by lowering the resistance of the sheath; that is, by increasing its permeability to ions by favouring inversion to an oil in water emulsion, and calcium would favour the change in the opposite direction. Clowes (1916) has studied such permeability changes and ion antagonisms with olive oil and water emulsions. More recently Dixon and Bennet-Clark (1930, 1932) have demonstrated that when water in olive oil emulsions are stimulated electrically, often the electrical resistance falls to a fraction of its initial value owing to a temporary inversion, and then it recovers. Moreover, they have shown that it is possible to obtain strength-duration curves with these emulsions; also that their sensitivities may be altered by various ions. To suppose that the membrane bounding nerve is essentially an emulsion would be in keeping with the observations just mentioned. A temporary inversion of the water in oil emulsion would explain the leakage of potassium from stimulated nerve. The depression of the injury potential could be explained as in (*a*) by the accumulation of some metabolite which possibly after long continued asphyxia might be present in an amount sufficient to cause inversion of the emulsion membrane thus permitting the escape of potassium salts. Oxidative removal of such a substance would restore first normal permeability and subsequently the injury potential.

My sincere thanks are due to Professor A. V. Hill for his continued interest and advice during the course of this work; to Professor J. C. Drummond in whose laboratory some of the preliminary analyses of crab's blood were done; to Dr. E. J. Allen, the Director, and to the staff of the Marine Biological Laboratory, Plymouth, for their help and courtesy during my visits there; to Professor C. M. Yonge (formerly a member of the staff of the Marine Biological Laboratory) who took for me the first series of samples of *Cancer* blood and nerve mentioned; to Professor R. Höber for helpful criticism during the preparation of this paper; to Mr. J. L. Parkinson for his willing assistance at all times.

I am also indebted to Professor A. V. Hill for the loan of apparatus; to the Government Grant Committee of the Royal Society and to the Thomas Smythe Hughes and Beaverbrook Funds of the University of London for grants which have defrayed in part the cost of apparatus and materials.

Summary.

(1) Samples of blood serum and nerve from *Cancer pagurus* have been analysed for total solids, inorganic cations, and potassium.

(2) Similar analyses have been made of serum and nerve from *Maia squinado.*

(3) A modification of Kramer's method of estimating the potassium in 1 c.c. samples of blood plasma or serum is described. It is designed to give an accuracy of ± 1%, and to save time when a large number of samples has to be analysed.

(4) The usual value obtained for the injury potential of *Maia* nerves was about 30 millivolts. The "crush sealing over" effect described by Gerard (1930) was very pronounced, but if precautions were taken to obviate this the injury potential was reproducible to within ± 5% of its value for about 2 hours.

(5) Over a period of about 8 hours in oxygen at 17° C. the injury potential of *Maia* nerve declined at a rate of 3–4% per hour.

(6) Potassium chloride rich solutions depressed both the action current and the injury potential rapidly and reversibly. The minimum potassium concentration required to abolish completely the action current was about five times that occurring in sea water or in *Maia* blood.

(7) The depression of the action current by potassium rich solutions could be partly prevented by the addition of calcium chloride.

(8) The injury potential of a nerve could be reduced from 30 millivolts to 3·5 millivolts if the concentration of potassium chloride in the fluid bathing the outside was made equal to the apparent potassium concentration (as determined by chemical analysis) inside the nerve. With rather higher potassium concentrations the injury potential could be made zero. Over a limited range an approximately linear relation was found to hold between the depression of the injury potential and the logarithm of the potassium concentration.

(9) Nerves dehydrated in hypertonic solutions could be made to give increased injury potentials.

(10) It has been shown that the reversible inexcitability which occurs in freshly dissected crab's nerve is probably due to the presence of an abnormal concentration of potassium salts at the outside surfaces of the fibres.

(11) Like potassium, rubidium and cæsium ions could abolish reversibly the action current in *Maia* nerves, whilst the injury potential was affected little. Ammonium ions caused inexcitability, but there is some doubt as to whether they act in precisely the same way as the alkali metal ions.

(12) The action of rubidium or cæsium ions in depressing the action current could be partly prevented by calcium ions.

(13) Resting nerves in sea water were found to lose small quantities of potassium salts or none at all, but quite considerable quantities escaped from nerves stimulated to fatigue.

(14) Nerves which had been asphyxiated for a short time could be restored by the admission of oxygen, but nerves which had been asphyxiated for a longer time also required washing with sea water for complete recovery. Possibly potassium salts leak out in the latter case.

(15) If it is assumed that the potassium, which has escaped from a stimulated nerve, is restored by a secretory process, then the thermodynamical efficiency of crab's nerve can be calculated to be about 0·02.

(16) Two possible physical mechanisms intended to explain the observations summarized above are discussed.

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Physico-Chemical Studies of Complex Organic Molecules. Part I.—*Monochromatic Irradiation.*

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[Plates 7 and 8.]

§ 1. Theoretical Considerations.

The physical study of polyatomic molecules has proved to be a task of great difficulty. It now appears likely that, by combining observations of infra-red absorption spectra with observations of Raman emission spectra, and by making use of several subsidiary relationships, accurate information can now be given as to the resistance both to stretching and to bending of all the important links of molecules such as C_2H_2, C_2H_4, C_2H_6, CH_2O, and N_2O_4.* But it is evident that a limit has nearly been reached and that molecules of greater complexity present to the physicist an almost insoluble problem.† Thus, whilst we may perhaps hope for a complete analysis of the structural elements of acetone, in the not very distant future, a similar analysis of methyl ethyl ketone seems at present to be impossible. *A fortiori*, the complex molecules which are of so much importance in biology, are entirely beyond the scope of these methods of examination.

Nevertheless much valuable information may be obtained by the application of physical methods, in view of the fact that the mutual influence of the binding electrons is limited to a very narrow range, and is usually confined (even for strongly polar radicals) to not more than about three atoms of a long chain. While, therefore, we cannot hope to provide a complete *mechanical* description of such a model, in terms of its *vibration* spectrum, we may be able to elucidate its *electronic* states with some ease and certainty (Mulliken, 1933). Thus the long-established chemical practice of regarding the absorption band at about 3000 A., which is characteristic of all ketones and aldehydes, as caused by an electronic transition in the $>C=O$ group, is justified by the new orbital theory and by more precise experiments, with the qualification that any attempt to localize the excitation in the electrons of the double bond itself is definitely not valid. For all $>C=O$ groups in the " aldehydic " or " ketonic " environment there will be an energy of excitation of about 4 volts, corresponding to the band at 3000 A.; this energy of excitation will raise the group (and hence the molecule) to its first excited state. This first excited state will be at the same energy level through the series of molecules above, but will be much modified in different environments, *e.g.*, by the proximity of $-OH$ or $-NH_2$ in $-CO.OH$ or $-CO.NH_2$ or by the presence of conjugated double bonds, *e.g.*, $-C=C-C=O$.

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* For the first four molecules, *cf.* Mecke (1932), and Sutherland and Dennison (*in course of publication*), and for N_2O_4 *cf.* Sutherland (1933).

† The obvious method of identifying a Raman line of given frequency with the vibration of a particular type of bond is not to be trusted without a more rigorous analysis of the complete vibration spectrum, *cf.* Fermi (1931), and Sutherland and Dennison (*loc. cit.*).

Similar considerations may be applied to the photochemistry of complex molecules. The initial absorption of a quantum of light by one of the more easily excited radicals in the molecule gives rise to an excitation, which is at first mainly confined to the valency electrons of this radical, since the other electrons are not directly concerned. The excited electron may then revert to the ground state from which it was raised; but, if the excited state possesses sufficient energy to overcome the stability of the bonds, it may give rise to a decomposition, which need not be confined to the excited radical but may extend throughout the molecule. At first sight, therefore, it might appear that the photochemical decomposition of a complicated organic molecule would be a disorderly process, like the thermal decompositions which occur when a non-volatile organic material is charred. Recent work, by Norrish and his colleagues (1934) specially, has shown, however, that photochemical decompositions may pursue an orderly course, even when the excited radical forms a part of a large molecule. Thus when an aldehyde is decomposed with elimination of carbon monoxide, the radicals on either side reunite in an orderly way to form a product which may under favourable conditions be almost entirely homogeneous. Whereas, therefore, the thermal decomposition of a complex molecule is often accompanied by far-reaching destruction, it is possible for a photochemical decomposition to proceed so smoothly that a relatively drastic operation is followed by a complete healing of the local wound.

The purpose of the present paper is to indicate that operations of this kind can only be conducted to the best advantage by the process of monochromatic irradiation, and this for two obvious reasons. *First*, complex molecules often have a very complicated absorption spectrum, including absorption bands both in the visible and in the ultra-violet region. It is a task of some difficulty to sort out these bands, and to discover how many of them must be assigned to one radical, and how many to another. The bands may also differ widely in their photochemical sensitiveness. It is therefore essential, when working with molecules of this kind, to begin by producing a homogeneous excited state, by irradiating the molecule with light of a single wave-length, and thus activating one radical to a constant energy level, instead of several radicals to different energy levels. *Second*, the product of irradiation may have an even more complicated spectrum than the original material, and in particular it may develop absorption bands in close contiguity to, or even overlapping, those of the original molecule. If complicated secondary changes are to be avoided, therefore, it is essential to shield the product from radiation which would produce a further excitation of the primary product. This can only be done by selecting, whenever possible, radiations which are absorbed by a particular radical of the original molecule, and not absorbed by the product.

Since these limitations are not merely theoretical, but are of urgent practical importance, considerable interest attaches to the provision of sources of monochromatic light, which shall be sufficiently powerful to bring about adequate chemical changes within a reasonable space of time. An apparatus for this purpose is described below and some examples are given to illustrate the way in which it may be employed.

§ 2. MONOCHROMATIC RADIATION.

It is difficult to obtain monochromatic ultra-violet light of sufficient intensity for photochemical reactions. Filters may be used in the visible and near ultra-violet with some success, but in the far ultra-violet they are unsatisfactory Most substances transmit in several regions of the spectrum so that a series of filters is necessary to give light in any one region. This diminishes the intensity considerably, and the light, when transmitted, is not monochromatic but covers a considerable range of wave-lengths. In addition, many filters are decomposed by ultra-violet light, so that the nature and intensity of the transmitted beam changes continuously.

Quartz Monochromator.

Because of these difficulties, a quartz monochromator was constructed, of sufficient size to give monochromatic light of the required intensity (Bowden, 1931).* The general design of the instrument is shown in fig. 1.† Light from the source L was rendered parallel by the collimating quartz lens A, passed successively through the two quartz prisms B, B′, and was brought to a focus on the radiation cell by the quartz lens A′.

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* We are greatly indebted to Dr. R. Fraser for assistance with the detailed design.

† For a description of various monochromators see Forbes (1928), and Heidt and Daniels (1932).

The large compound short focus quartz lenses A, A′ were each made up from two convex lenses 10 cm. in diameter cut from natural quartz. The usual working aperture was 1·8 for the 3650 A. line, but stops could be used to reduce this. The lenses were carried in telescope tubing and were focussed by the racks and pinions R, R′, the position of the lenses being shown on the graduated scales S, S′.

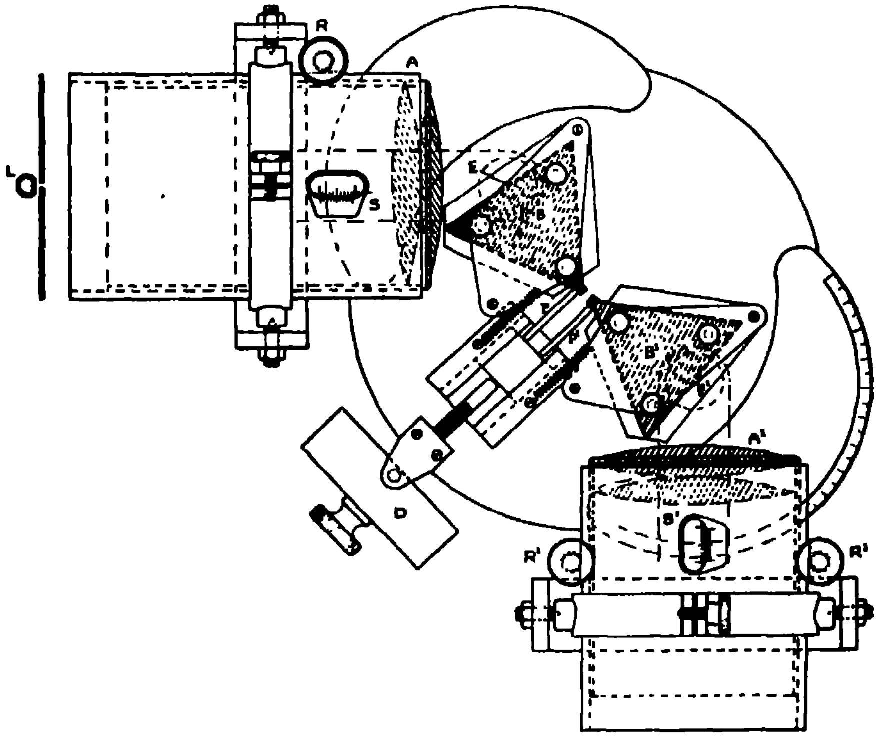

Fig. 1.

The 60° prisms (face 8 × 8 × 8 cm.) were each formed from single transparent crystals of natural quartz cut with the optic axis of the crystal parallel to the base of the prism. The difficulty of obtaining natural crystals of sufficient size was increased by the condition that one must be right handed and the other left handed so that they mutually correct for birefringence. The use of two 60° prisms in series gave a high dispersion, which was important since it enabled a wide slit to be used without overlapping of adjacent lines in the spectrum, and so gave a high total flux as well as a high intensity of monochromatic light.

Both the collimator and telescope were carried on pivoted arms which could be swung into any position, in order that the instrument could work at any deviation or be used with one prism or two. Since, in practice, it was inconvenient to move either the source or the irradiation cell, these arms were usually clamped in position, and light was moved on to the cell by rotating the prisms equally, in opposite directions, through a small angle. The prisms were carried on tables which could rotate about an axis in the centre of their outside faces at E and E′. The rotating movement was communicated to the prisms by the pins P, P′ which were, in turn, controlled by the fine pitch screw and wave-length drum D. In this way light of any selected wave-length could be moved on to the cell merely by turning the wave-length drum.*

The image of a straight slit is curved by optical distortion in the large prisms, so the slit was made of an appropriate curvature to give a straight image.

Source of Ultra-violet Light.

A standard mercury vapour lamp with a flattened side was used as a source, and gave a sufficiently high intensity for many purposes. The flattened side enabled the slit to be brought nearer the centre of the discharge in the lamp. The intensities of the lines in the far ultra-violet were improved by deflecting the discharge against the wall of the lamp by means of a magnetic field. With prolonged use, the quartz became more opaque to the far ultra-violet, and the intensity in this region fell off considerably, making it necessary to empty and clean the lamp at intervals.

The most satisfactory type, however, was a small capillary lamp in which a current of several amperes passed through mercury vapour in a capillary tube 1 to 2 millimetres in diameter. No slit was used on the monochromator, the capillary tube itself acting as a slit. Several forms of those lamps have been described (Daniels and Heidt, 1932); they give a high intensity but the quartz becomes relatively opaque to the far ultra-violet in a few hours, and a frequent renewal of the capillary is necessary. A very simple form which could be made and fitted in a few minutes was devised by Mr. A. J. P. Martin and Mr. S. D. D. Morris, so that the trouble of replacement was reduced to a minimum.

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* The mechanical parts of the instrument were constructed by W. G. Pye & Co., Cambridge.

The general construction of this lamp is shown in fig. 2. The lamp itself, which is a horizontal one, was made from quartz tubing 1 to 2 mm. in internal diameter, in which were blown two small bulbs (2 to 3 cm. apart). The tube was filled with dry mercury and the iron wire electrodes sealed in by means of cycle valve rubber tubing. The arc was struck between the bulbs by means of a small heating coil which pressed against the capillary tubing. The cooling jacket was made in two halves and the faces ground flat and greased so that when screwed together, with the lamp inside, it was water tight. Distilled water was neccasary for cooling to prevent deposition of lime on the outside of the lamp.

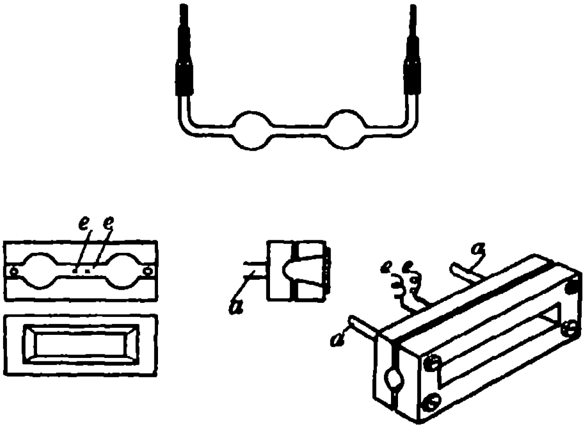


FIG. 2.

In using the mercury lamp one is, of course, restricted to the lines present in the mercury spectrum, although additional lines can be obtained by adding other metals such as zinc and cadmium to the mercury. A hydrogen lamp does not suffer from this disadvantage, since it gives a continuous emission extending far down into the ultra-violet. A large hydrogen lamp similar to that described by Lawrence and Edlefson (1930) to carry 4 amperes at a few millimetres pressure was constructed. This gave a high intensity of continuous ultra-violet light so that radiation of any wave-length can be selected by the monochromator. An all quartz lamp to carry 8 amperes at 6000 volts is under construction.

Intensity of Monochromatic Light.

The intensity of the different lines of the mercury spectrum transmitted by the monochromator is shown in fig. 3. The source was a capillary lamp which had been running continuously for an hour.

It will be seen that the intensity is extremely high. For the 3650 line the intensity is over a million ergs. per second per square centimetre. The usual sort of intensity employed in photochemical reactions with filtered light in the 3650 region is *ca.* 2×10^3 ergs. per sec./cm.^{2}. The image of the line was 0·15 cm. wide by 3 cm. long (area 0·45 cm.), so that the total flux of energy in the 3650 line was *ca.* 5×10^5 ergs. per second. The intensity obtained by the monochromator ($1{\cdot}3 \times 10^6$ ergs./sec./cm.^{2}) corresponds to *ca.* 2×10^{17} quanta sec./cm.^{2} of the 3650 radiation, and the total flux to *ca.* 8×10^{16} quanta per second.

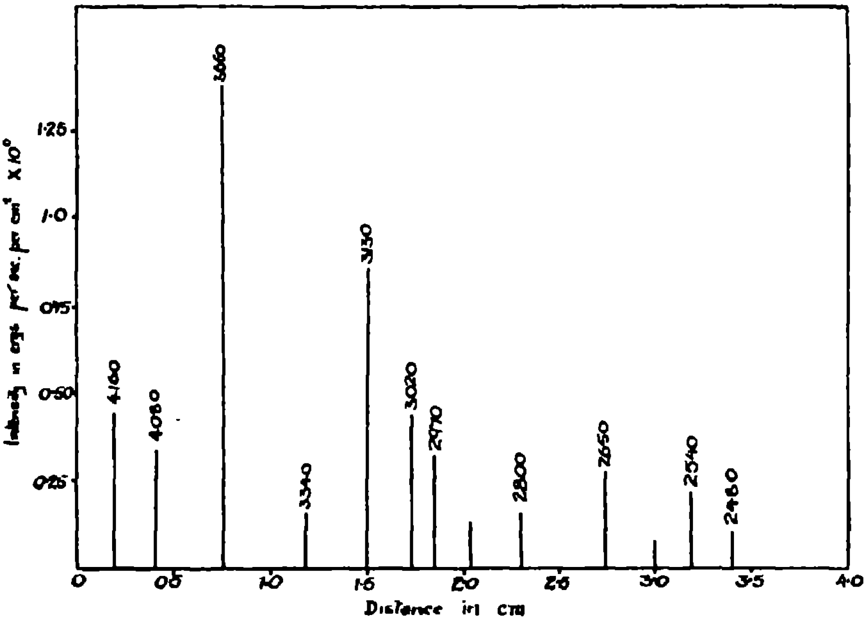


FIG. 3.

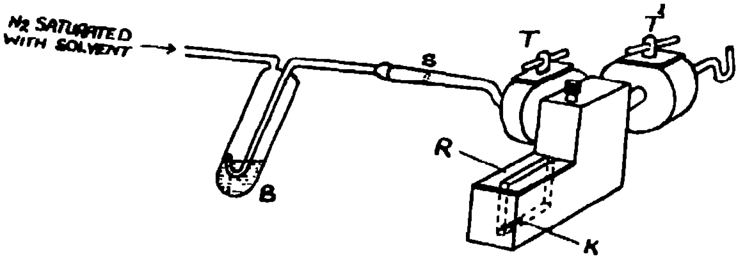


FIG. 4.

Irradiation Cell.—One of the irradiation cells used is shown in fig. 4. It consisted of a rectangular slot R cut in a brass block and fitted with quartz windows at the top and bottom. The width of the cell was made equal to the width of the slit image (usually 2 mm.) so that the whole of the cell was covered by the radiation. The quartz windows were attached with a thin film of hardened seccotine, or were soldered directly on to the brass. This was done by first platinizing the quartz and tinning with soft solder and Woods metal. Cells made wholly of quartz have also been used.

The cell was fitted with steel taps T, T′, which were mercury sealed, so that the cell was gas tight, and irradiation could be carried out in the absence of air. The cell was filled through the side tube S; this was connected by an accurately ground conical joint to the small bottle B containing the air free solution. By suitably rotating the bottle B about this conical joint, first nitrogen, and then the solution, were blown into the cell. The taps T, T′ were then closed and the bottle removed. The absorption spectrum of the solution could be examined before and after irradiation without removing it from the cell. The solution was stirred continuously by means of small steel shot K which were kept in motion by an intermittent electromagnet fixed near the cell.

The intensity of the light was measured on a Moll thermopile fitted with a rectangular slit of the same cross-section as the cell. By turning a screw the thermopile could be moved under the radiation in place of the cell, and the intensity of the light falling on the cell could be measured at any instant. Fig. 5, Plate 7, illustrates the monochromator in the vertical position, with the cover removed, and shows the general arrangement of the capillary lamp L, the irradiation cell C, the thermopile T and the stirring magnet M.

Measurement of Absorption Spectra.

The absorption spectra in the ultra-violet were photographed on a small Hilger quartz spectograph using a hydrogen lamp as source of illumination. The small dispersion of this instrument and the continuous source made it possible to detect weak bands and fine structure which might be overlooked on an instrument of larger dispersion or with a line source. The absorption coefficients were measured quantitatively on a Hilger E2 spectrograph, using either a polarization photometer, or the new Hilger echelon cell.

§ 3. Ergosterol and Vitamin D.

The transformation of ergosterol into calciferol (vitamin D) is one of the best known "biological" photochemical changes, and was clearly a good substance for the first application of monochromatic irradiation (Bowden and Snow, 1932).

Absorption Spectra of Ergosterol and Calciferol.

The well-known absorption spectra of ergosterol and calciferol were re-measured in the solid state, and in solution in cyclohexane. In cyclohexane ergosterol has clearly marked bands with maxima at 2940, 2820, 2720, 2600 A. and calciferol a broad, diffuse, and very strong band with its maximum at 2650 A. in close general agreement with earlier observations (Askew and others, 1932).

The Effect of Monochromatic Radiation upon Ergosterol and Calciferol.

It has been known for some time that irradiation of ergosterol by ultra-violet light of wave-lengths longer than 2750 A. gave a better yield of calciferol than light of shorter wave-lengths and that calciferol is easily destroyed by ultra-violet light, especially of short wave-lengths (Bourdillion and others, 1931).

(*a*) *The Irradiation of Calciferol.*—The absorption spectra of calciferol, and the changes occurring in it on irradiation with light of different wave-lengths are shown in fig. 6, Plate 8, photographs 1, 2, 3 and 4. Dark bands correspond to absorption.

λ 3130 and wave-lengths longer than this produced no appreciable change, photograph 2.

λ 2650, destroyed the absorption completely and quickly, photograph 3.

λ 2537 also destroyed the absorption less quickly than λ 2650, photograph 4.

The destruction of calciferol by irradiation was very little affected by working in an atmosphere of nitrogen instead of in air.

(*b*) *Irradiation of Ergosterol.*—Since calciferol is readily destroyed by λ 2650 it is apparent that only a small region of wave-lengths can transform ergosterol into calciferol without destroying a large fraction of the latter. The overlapping of the absorption curves makes these narrow limits of effective wave-lengths inevitable. Photographs 5, 6 and 7 in fig. 6, Plate 8, show how sharp is the long wave-length limit.

λ 3130 caused no change even after many hours in an atmosphere of nitrogen, photograph 6.

λ 2967 caused a destruction of the ergosterol band, and in nitrogen a rapid development of the calciferol band at 2650, photograph 7.

[The experiment was stopped after 2 hours, and the biological activity tested by Mr. M. W. Pirie. The result showed that 60% of ergosterol had been transformed into the vitamin, but too few rats were used to make this figure reliable and there is a possible error of ± 30%. Since no particular care was taken to ensure a maximum yield, it seems that efficient conversion may be possible by this wave-length. The best yield obtained by Askew *et alii* with filtered light and a continuous flow was 44%.]

λ 2650 destroyed the ergosterol, but produced a smaller yield of calciferol.

In this photochemical reaction (ergosterol → calciferol) the supply of oxygen plays an important part. In the presence of oxygen, ergosterol is readily destroyed, but gives a product which is transparent down to 2400 A, photograph 8, fig. 6, Plate 8.

The quantitative analysis of these photochemical reactions has not yet been attempted. It is apparent, however, that the quantum efficiencies and orders of the reactions could be obtained by this technique in a way quite impossible with light which is not monochromatic (Marshall and Knudsen, 1930).

Irradiation of the Solid Crystals.

The absorption spectra of thin crystals of ergosterol and calciferol was found to be the same as in solution except that the heads of the bands were slightly displaced toward longer wave-length. The crystals were subjected to monochromatic irradiation and the changes which occurred in the optical and X-ray properties were measured by Bernal (1932). Examination under a polarizing microscope showed that radiation with an effective wave-length could transform the crystal from anisotropic to isotropic material. At the same time the X-ray pattern was destroyed. Ergosterol was scarcely affected by light of wave-length 3650 A., 3130 A. or 2537 A. but was rapidly transformed presumably into calciferol by light of wave-length 2967 A. Calciferol on the other hand was stable to 3650 A. and 2537 A. (which destroyed the solution) but was destroyed by 3130 A. (to which the solution was stable). This difference in behaviour of the solid and the solution is due partly to the shift in the bands and partly to the greater absorption and the formation of protective absorbing layers on the surface.

§ 4. VITAMIN B_1.

No definite absorption bands had been attributed to the molecule of vitamin B_1 at the time of this investigation. Damianovich (1922) and later Guha (1931) found that active yeast concentrates possessed a band in the region of 2600 A. but it was shown by Dixon, Keilin and Guha that inactive fractions also gave this band. Mukerji observed that crystals of the active substance, obtained from rice polishings, gave an absorption at 3900-3300 A. Guha concluded that it was unlikely that the study of absorption spectra of vitamin B_1 concentrates would yield any significant results. On the other hand Windans and his collaborators (1932) have published an absorption spectrum of crystalline vitamin B_1 with a maximum at 2600 A.

The specimen of vitamin B_1 (for which we are indebted to Mr. T. W. Birch, who originally obtained it from Jansen and Donath) showed absorption bands at 2600, 2400, 2100 A., fig. 7, Plate 8, photograph 1. The last two bands have not reproduced well on the print. Since the crystals were impure, it was not known whether any of these bands could be associated with the vitamin molecule.

Irradiation for 1 hour with λ 2537 (which falls within the first band only) destroyed the band at 2600; and left the bands at 2400 and 2100 A. unaffected, photograph 2, fig. 7, Plate 8. The irradiated substance, with the 2600 band almost gone and the other bands intact, was tested for biological activity by Mr. Birch. Almost all of the activity was destroyed.

It became probable that the 2600 band was characteristic of the biologically active molecule; and either all the bands are due to the same molecule as 2600, but depend upon a group which is unaffected when the 2600 is destroyed, or else the short wave-length bands are not produced by the same molecule as the 2600 band.

The conclusion that vitamin B_1 possesses an absorption at 2600 A. is inevitable unless we assume the hypothesis of a secondary photochemical change. The molecule responsible for the 2600 band could be different from the vitamin molecule originally, but might become photochemically activated or dissociated by the irradiation and so react with the vitamin molecule. If that occurs, the disappearance of the 2600 band would not prove that the band was characteristic of the vitamin.

The complication of a secondary photochemical change is unlikely under the conditions of the experiment, and is particularly improbable for so stable a molecule as vitamin B_1. Further experiments argue against it. An effective way of destroying B_1 activity by chemical method is to heat it with an alkali; a specimen thus treated, and with its B_1 activity destroyed, was examined spectroscopically. It showed a similar absorption spectrum to the irradiated product, photograph 3, fig. 7, Plate 8, the band at 2600 had again disappeared and the bands at 2400 and 2100 remained.

A correlation of vitamin B_1 activity with absorption at 2600 was therefore established. This does not mean that the *head* of the band is necessarily situated exactly at 2600. More detailed irradiation experiments over narrower limits would be necessary to decide this but it does show that vitamin B_1 must possess a strong absorption at λ 2600. More recently Heyroth and Loofbourow (1932) have measured the absorption at 2600 in a large number of vitamin B_1 concentrates and conclude that it is characteristic of the vitamin. In a still later paper Peters and Philpot (1933) find that a new and stronger preparation (Kennedy and others, 1933) has an absorption maximum at λ 2450 with a "slight hump" at λ 2600. They do not find, however, any marked correlation between the 2450 band and the activity. Their absorption spectrum bears a general resemblance to the spectrum we observed for the Jansen and Donath preparation except that in the latter the "hump" at 2600 was more pronounced.

§ 5. VITAMIN A.

The characteristic bands of vitamin A and its biological precursor carotene have been known for some time. Vitamin A has apparently only one band, at 3280 A. which is connected with the vitamin molecule itself. Carotene in cyclohexane has a well-marked band system in the visible with maximum at 4860, 4540 and 4280 A. and a band in the ultra-violet at 2700.

Irradiation of Vitamin A.

A concentrate of vitamin A was irradiated by λ 3130. The band at 3280 A. quickly disappeared and the product was transparent down to 2000 A.

Heilbron and Morton had already observed that the full light from a mercury lamp destroyed the band at 3280 A.

Irradiation of Carotene.

Carotene was irradiated by 2650 for 2 hours in an atmosphere of nitrogen. The carotene band was partially destroyed and a new band developed in the region of 3280 A. (Bowden and Snow, 1932). Under the conditions of the experiment, the band was indistinguishable from the 3280 band of vitamin A; the solution was tested with antimony trichloride and gave the Carr-Price increase of the colour ratio of blue to yellow. The substance produced by irradiating carotene, the known biological precursor of vitamin A, thus gave the two accepted *physical* tests for the vitamin. The facts were striking, and it was obviously important to apply rigorous tests to decide whether the product was identical with vitamin A.

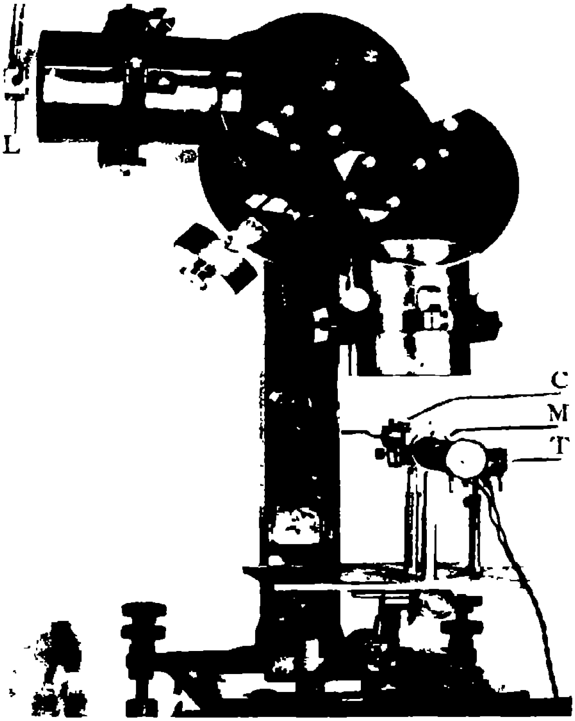
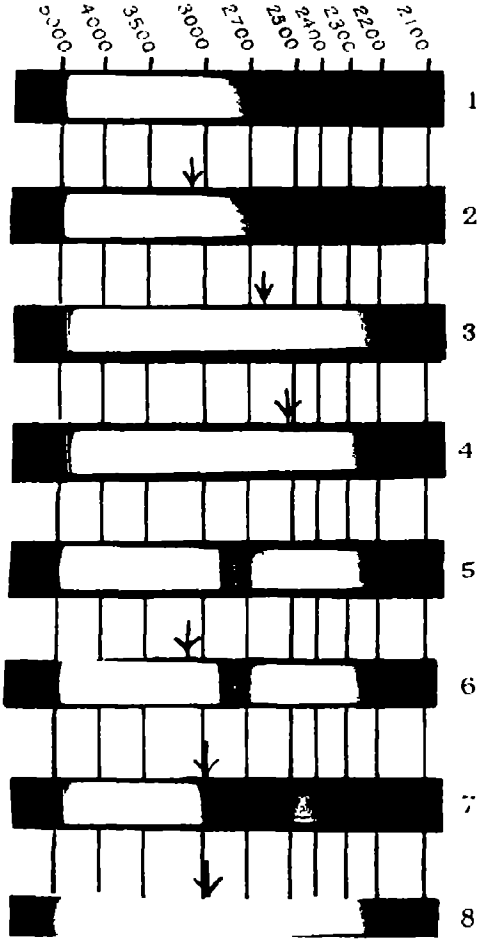


Fig. 5.

(*Facing p.* 272.)

FIG. 6.



Calciferol.

Calciferol irradiated by λ 3130 A for 1 hour.

Calciferol irradiated by λ 2650 for 30 minutes.

Calciferol irradiated by λ 2537 for 1 hour.

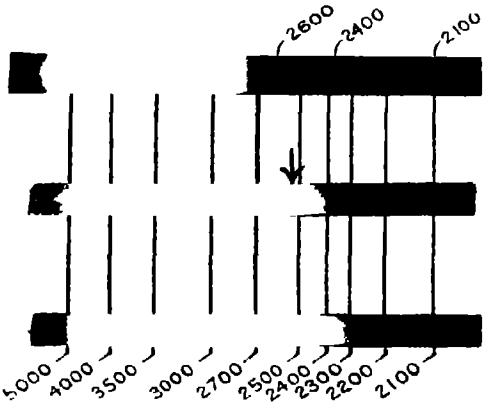
Ergosterol.

Ergosterol irradiated by λ 3130 for many hours

Ergosterol irradiated by λ 2967 A for 2 hours in nitrogen atmosphere.

Ergosterol irradiated by λ 2967 for 10 hours in oxygen atmosphere.

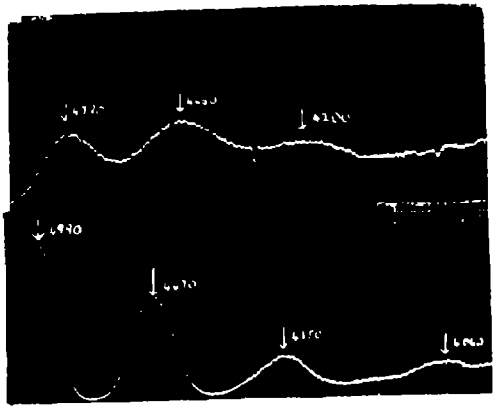
FIG. 7.



1. Vitamin B_1.

2. Vitamin B_1 deactivated by irradiation with λ 2537

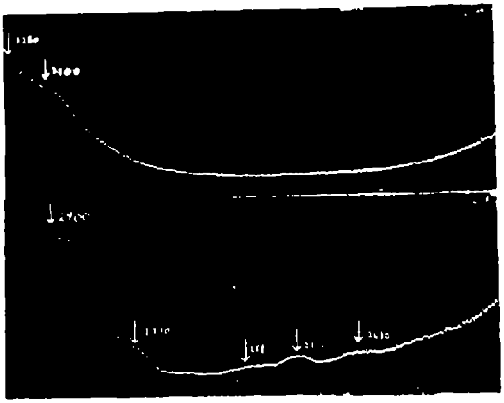
3. Vitamin B_1 deactivated by boiling with alkali.



1 Carotene.

(*a*) Room temperature.

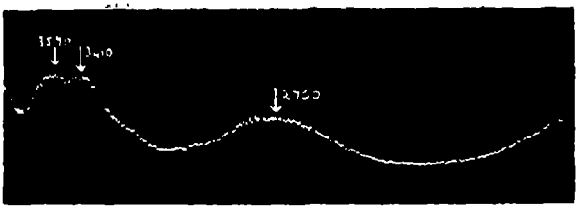
(*b*) Liquid air temperature.



2. Vitamin A

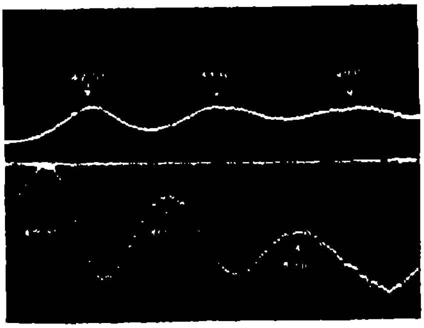
(*a*) Room temperature

(*b*) Liquid air temperature.



3. Irradiated carotene

Liquid air temperature



4. Pigment from vitamin E concentrate

(*a*) Room temperature.

(*b*) Liquid air temperature.

Clearly, the only ultimate test for the *biological* activity of a molecule is its action on animals. For carotene and vitamin A, the biological test is particularly difficult, since carotene itself is converted into vitamin A in the body of the animal. This difficulty was pointed out by Heilbron and Morton (1932) who also advanced reasons why the irradiation product could not be identical with vitamin A. In the circumstances it was desirable to develop more stringent physical criteria to apply to the irradiated product and to vitamin A, in order to test whether they were the same, before resorting to the biological test.

We wish to express our thanks to the Royal Society for a grant to one of us (F. P. B.) for the monochromator.

Summary.

A method is described for the production of monochromatic light of sufficient intensity to bring about reasonably rapid photochemical changes. The irradiation can be performed on very small amounts of material and the progress of the reaction followed spectroscopically. Selective monochromatic irradiation is applied to some of the large molecules of biological importance notably ergosterol and calciferol, vitamin B_1, carotene and vitamin A.

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Physico-Chemical Studies of Complex Organic Molecules. Part II.—*Absorption Spectra at Low Temperatures.*

By F. P. Bowden and S. D. D. Morris, Laboratory of Physical Chemistry, Cambridge.

(Communicated by T. M. Lowry, F.R.S.—Received December 27, 1933.)

[Plate 9.]

The physical criterion which has proved most valuable in distinguishing between different complex molecules is the absorption spectrum, but the absorption spectra have nearly always been measured in solution at ordinary temperatures, and usually give broad structureless bands. These bands frequently appear similar for different molecules, and are obviously unsatisfactory for the purpose of recognizing a particular molecule and distinguishing it uniquely. The ideal data for spectroscopic recognition are lines which are peculiar to a given atom or molecule.

At low temperatures the bands of many molecules develop a structure with a considerable amount of detail (Kronenberger, 1930; Conant and Crawford, 1930; Arnold and Kistiakowsky, 1932; Spedding and Bear, 1933; Robertson, Fox, and Martin, 1934). This is probably due to two main factors: (i) the ground state of the molecule is simplified at low temperatures by the elimination of all but the vibrational states of lowest energy; and (ii) the Stark effects of the molecular fields of neighbouring molecules is rendered more constant as the molecules become quiescent, consequently the blurring is reduced. Both effects tend to diminish the width of the absorption bands and to bring out details of structure which cannot be recognized at atmospheric temperature. The development of structure will not be a property of all molecules; those, whose upper state of the electronic transition corresponds to dissociation or to "predissociation," will have bands continuous under all conditions. But for a very large number of molecules, it should be possible to obtain structure at low temperatures, and in all cases narrowing of the bands should occur.

Liquid Air Technique.

In order to observe the absorption spectrum of a dissolved substance at low temperatures, it is essential that the solvent should not give an opaque crystalline mass when frozen, but should remain transparent. Several solvents have been used, but a very suitable one is ethyl alcohol, which solidifies to a transparent glass when cooled quickly.

The apparatus for freezing the solution and measuring the absorption spectrum at temperatures near that of liquid air is shown in fig. 1.

The solution was held in the small cylindrical cell C made of heavy brass and fitted with quartz end plates. A heavy copper rod R connected the cell to a copper tube T which was sealed on to the outside glass vessel. Quartz windows were attached to the short side arms A and the interior of the vessel was silvered to diminish radiation. The top of the cylinder was closed by a ground glass stopper carrying a tube for liquid air.

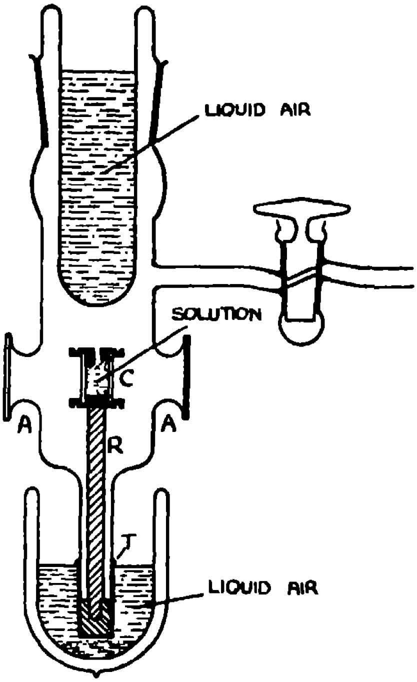

FIG. 1.

After filling the cell with the solution, liquid air was first poured into the stopper so as to condense the water vapour and carbon dioxide present in the vessel and prevent them from fogging the quartz windows. The copper tube was then immersed in liquid air and, after allowing time for the alcohol to cool to a viscous liquid, the vessel was evacuated. The alcohol solidified and was cooled to a temperature near that of liquid air.

Absorption Spectra of Vitamin A, Carotene and Irradiated Carotene at Liquid Air Temperature.

Under these conditions of freezing and cooling to a low temperature a solution of carotene in ethyl alcohol showed a marked sharpening of the three bands in the visible and the development of a new band (Bowden and Snow, 1932). The effect is shown in photographs 1 (*a*) and 1 (*b*), Plate 9. Photograph 1 (*a*) at room temperature shows the carotene bands at 4790 A., 4440 A., and 4200 A. Photograph 1 (*b*) shows the effect of cooling. The bands have sharpened very considerably and have been displaced toward longer wavelengths to 4990 A., 4670 A., and 4350 A., while a fourth band has appeared at 4060 A.

The ultra-violet band of carotene at 2700 A. became sharper at low temperatures, but it was little displaced and showed no structure.

The absorption spectrum of a vitamin A concentrate* at room temperature is given in photograph 2 (*a*) and shows part of the characteristic 3280 A. band. This band is diffuse, but in all the concentrates we have examined it has a small but definite subsidiary maximum at 3100 A. The effect of cooling to liquid air temperature is shown in photograph 2 (*b*). The main 3280 A. band is shifted to 3350 A., and new bands appear at 2900 A., 2770 A., 2580 A., 2510 A, and 2430 A. The last three were of low intensity. They can all be seen more clearly in the original plates than in the photometrical record.

Identically the same structure at low temperatures was found in two different B.D.H. concentrates and in a concentrate from turbot liver oil prepared by Dr. T. Moore ($_{1\,\mathrm{cm.}}^{1\%}\mathrm{E} = 1000$ approx). It is possible that the detailed structure may be due to an impurity associated with vitamin A. This possibility cannot be excluded from any spectroscopic observation not made with pure substances.

The effect of irradiating carotene in its ultra-violet band at 2700, using monochromatic light (λ 2650), was to cause the development of a new band in the region of 3280 A. which at room temperature closely resembled the vitamin A band.

The effect of cooling to liquid air temperatures, however, showed that there was a marked difference between them. The new band was shifted to longer wave-lengths and broke up into a series of bands at 3780, 3570, 3410 and 3210 A. This can be seen in photograph 3, Plate 9. The remnant of the carotene band at 2700 is still present and the new band shows structure. The 3210 A. band is faint and has not reproduced on the photometered record. The 3780 A. band is beyond the limit of the photograph. It is clear, however, that the detailed structure at low temperature is quite unlike that of vitamin A at low temperature. This more precise spectroscopic test shows that the molecule produced by irradiating carotene is different from that associated with the band at 3280 A. in vitamin A concentrates.

Vitamin E Concentrates.

Working in collaboration with Dr. T. Moore measurements have been made on the absorption spectrum of wheat germ oil and the non-saponifiable fraction from it which are known to contain vitamin E. Evans and Burr (1927) showed that the concentrate possessed a high general absorption but concluded that it did not give any definite bands that might assist in following the further concentration of the vitamin. We found that the bands given by the wheat germ oil were so numerous and diffuse that it was difficult to disentangle them. At the temperature of liquid air, however, the broad diffuse bands became sharper and many of them developed a structure so that the task of analysing and allotting them was rendered very much easier. The spectrum of the concentrate (the non-saponifiable fraction) is obviously due to several different molecules. Some progress has been made in separating these different fractions and their absorption spectra have been described (Bowden and Moore, 1933, *a*, *b*; Morton and Edisbury, 1933; Euler, Heelstrom and Klussmann, 1933). An example of the sharpening of the absorption bands at low temperatures is given in photograph 4, Plate 9, which shows the absorption spectrum of the coloured pigment extracted from the concentrate. It will be seen that there is a marked improvement in the definition of the bands.

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* A British Drug House concentrate $_{1\,\mathrm{cm.}}^{1\%}\mathrm{E} = 1000$.

The effect of cooling has always caused a sharpening of the bands and frequently a development of structure, and a shift toward longer wave-length. Accompanying this there is an increase in intensity of absorption.

We are very much indebted to Professor T. M. Lowry for his constructive advice and interest in this work, and to the Department of Scientific and Industrial Research for a maintenance grant to one of us (S. D. D. M.).

Summary.

The absorption spectra of some important biological molecules have been measured at liquid air temperature. The bands of β carotene (in ethyl alcohol) become narrower and shift to 4990 A., 4670 A., and 4350 A., and a new band appears at 4060 A. The ultra-violet band at 2700 A. becomes sharper but is little displaced.

The main band of vitamin A concentrates at 3280 A. is shifted to 3350 A. and new bands appear at 2900 A., 2770 A., 2580 A., 2510 A. and 2430 A.

The irradiation product of carotene whose absorption band at room temperature resembles that of vitamin A develops a structure at low temperatures with bands at 3780 A., 3570 A., 3410 A. and 3210 A. This more precise spectroscopic test shows that the two substances are not the same.

The absorption spectrum of vitamin E concentrates is due to several different molecules and some progress has been made in separating these out.

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Factors influencing the Growth of Normal and Malignant Cells in Fluid Culture Media

By R. J. Ludford, Stroud Laboratory of the Imperial Cancer Research Fund,
Mill Hill, London

(Communicated by J. A. Murray, F.R.S.—Received March 3, 1934)

[Plates 10–14]

1. *Introduction*

In a former communication (Ludford, 1933,) attention has been directed to differences in the growth of mouse tumours in plasma and serum. With the aim of elucidating the significance of these differences, further series of experiments have been carried out, which have brought to light new facts necessitating the revision of my former conclusions, as well as those of previous investigators who have worked with fluid media. At the same time further insight has been gained into the complexity of the factors which influence the growth of tumours in serum. It has been found that different strains of transplantable tumours exhibit individual differences when grown in serum, and even tumours of the same strain show marked differences from time to time. Also, both the type of growth and its extent are influenced by the source from which the serum is derived, that is, whether it is homologous or heterologous. In describing these experiments it will be necessary therefore to deal with different strains of tumours separately.

2. *Mouse Sarcoma* 37

Concerning this strain of tumour I am unable to add anything to the account published in my former paper (Ludford, 1933). Neither in mouse serum, nor in rat serum has it been possible yet to obtain good outgrowths of the sarcoma cells. Pure serum and diluted serum; serum, with and without, embryo extract; serum from young and old rats, male and female, both pregnant and non-pregnant; also different types of cover slips have all been tried without influencing the type of growth.

In a few cultures small sheets of fibroblasts have grown out after some days. These cells are distinguishable from the sarcoma cells by the manner in which they (the former) segregate acid dyes such as trypan blue; also by their appearance by dark ground illumination. The cytoplasm of healthy fibroblasts is clearer than that of sarcoma cells, and the mitochondria appear more filamentous and coarser.

3. *Crocker Mouse Sarcoma*

This tumour also has defeated my attempts to obtain good outgrowths of malignant cells in serum. In the paper previously referred to, I noted that occasionally a few sarcoma cells adhered to the cover glass. Less than a dozen out of nearly a thousand cultures have shown sarcoma cells well spread on the cover glass. This occurred in old cultures which had not been subcultured for some days. Most of the sarcoma cells were much enlarged and many had three or more nuclei. Examined by dark ground illumination these cells presented a remarkable appearance. Their cytoplasm was filled with granules in vigorous Brownian movement, unlike the cytoplasm of cells of the same sarcoma growing in a plasma culture, which shows little movement. It has therefore been concluded that the large spread cells in these serum cultures were in a degenerate condition, which rendered them adhesive to a glass surface.

4. *Mouse Sarcoma* 2529

This tumour had not previously been grown *in vitro*. When transplanted into mice it grows more slowly than the Crocker sarcoma, or 37 S. It differs from these two sarcomata in that it will grow as sheets of malignant cells in both rat and mouse sera. In our experiments good sheet growths of malignant cells have been obtained in rat serum, when the polyblasts have become rounded or degenerate, or after subculturing several times so as to get rid of the polyblasts. Thus a marked difference has been found between cultures in which the serum has been renewed, and others which have been subcultured by removing the explant to a fresh cover glass and adding fresh medium. Fig. 1, Plate 10, represents the result of six-days-growth in rat serum. The explant was transferred to a fresh cover with more serum after one day, then again after two more days, and the culture was fixed three days later. The culture is an almost pure sheet of sarcoma cells, well flattened on the cover glass. Very few polyblasts remain, and they are rounded. Fig. 2, Plate 10, is a photomicrograph of another six·days-old culture of an explant of the same tumour. When the culture shown in fig. 1 was subcultured, that of fig. 2 simply had the old medium pipetted off and fresh added. Both cultures received the same medium at the same time. The p_H of two similar cultures was taken on the sixth day of growth, and found to be the same in both, about 7·6. Fig. 2 shows part of the extensive outgrowth of polyblasts, which extends outwards beyond the limits of the photograph. Only a few well-spread sarcoma cells (S) are to be seen.

Extensive outgrowths of polyblasts are obtained in plasma cultures of this tumour, and appear to interfere with sheet growth of the sarcoma cells also.

When trypan blue is added to either plasma or serum cultures of this tumour, the spread tumour cells are seen to segregate a considerable amount of dye. In this respect they differ from malignant cells of the same tumour growing *in vivo*, which, as I have previously described, do not segregate acid dyes as do fibroblasts.

5. *Mouse Carcinoma* 63

My former conclusion that this tumour would not grow as sheets of malignant cells in serum was wrong. I have since found that beautiful sheet growths can be obtained in pure, or diluted, mouse serum. Such growths cannot always be obtained, and appear to be dependent upon the state of the tumour. Since exactly the same technique has been employed in all my later experiments as in some of those previously described, I can only attribute my published results to the state of the tumour at the time those experiments were carried out. It has been clearly established that the growth of transplantable tumours fluctuates *in vivo* from time to time, and this is correlated with variations of growth *in vitro*; but even with tumours which have given excellent sheet growths of carcinoma cells in mouse serum, none has been obtained in rat serum.

I should point out here that I had accepted the views of previous workers without having ascertained myself, that mouse and rat sera were equally good culture media for mouse tumours, and that in most of my previous experiments rat serum was used.

Up to the present time approximately 3000 cultures of this tumour have been put up in rat serum, both undiluted and diluted, with various proportions of Ringer solution. In all but five cultures growth has been of the type described in my former paper (Ludford, 1933). Five out of twenty-four explants of one tumour in rat serum showed a tendency to form sheet growths. This was a tumour which grew most vigorously in mouse serum forming extensive sheets with innumerable mitoses, in twenty-four hours. The explants in rat serum, however, did not proceed to form perfect sheets of malignant cells. Only in one culture, fig. 3, Plate 11, was there a small imperfect sheet at one side of the explant forming a striking contrast to the expansive growths in mouse serum, fig. 4, Plate 11. Some sheet growths of malignant cells have also been obtained in a culture medium consisting of rat serum diluted with a hypertonic Ringer solution containing ten times the correct proportion of KCl. The same tumour in a medium consisting of rat serum with Ringer solution showed no sheet growth of the carcinoma cells.

The difference in the type of growth in mouse and in rat sera appears to be due to some influence which the media exert on the adhesive properties of the carcinoma-cell membrane. The following experiment supports this view.

Twenty-four cultures of carcinoma 63 (189 A) were put up in mouse serum, and the same number in rat serum. After two days all the cultures were washed with two changes of Ringer solution. To half of those previously, grown in mouse serum a drop of freshly prepared mouse serum was added, and to the other half a drop of freshly prepared rat serum. The cultures previously grown in rat serum were treated in the same manner. The results of the experiment are given in Table I.

Table I—5.12.33. 63/189A

2 days growth in	Type of growth	2 days growth in	Type of growth	No. of cultures out of 6
Mouse serum	Sheet growths of carcinoma cells	Mouse serum	Increased sheet growth of carcinoma cells	6
		Rat serum	Collapse of sheet growths of carcinoma cells. Polyblasts remain	5
Rat serum	Outgrowth of polyblasts	Mouse serum	Sheet growths of carcinoma cells	4
		Rat serum	Increased outgrowth of polyblasts	6

That the carcinoma cells of the explant, which has been for two days in mouse serum, then for two days in rat serum, are not all destroyed has been shown by subculturing the explants again with mouse serum when fresh sheets of carcinoma cells have grown out. Sheets of carcinoma cells have also grown from the rounded explants which have been four days in rat serum, twenty-four hours after transferring them to mouse serum.

This experiment has been repeated with similar results, but in another experiment in which extensive growth of carcinoma cells had occurred in mouse serum, the change to rat serum did not bring about destruction of the sheets ; although after forty-eight hours these cultures showed greater collapse of the sheet growths than those which had remained in mouse serum all the time.

These experiments point a warning to the use of serum cultures for therapeutic experiments, for if the adhesion of the cells to the cover glass is altered so that the cells become detached, then deprived of a support they round off and degenerate. As previous investigators (see Fischer, 1930) have pointed out, if cells are not supplied with some sort of support they cannot grow *in vitro*.

Further support for this view is afforded by the fact that this tumour will grow as sheets of carcinoma cells, either in mouse plasma, or in rat plasma. The latter, with fowl plasma added to delay liquefaction, constitutes a good medium for obtaining large sheet growths of carcinoma cells, the fibrin of the clotted plasma supplying the necessary support for the carcinoma cells.

Some cultures of this carcinoma in rat serum have exhibited outgrowths of multinucleate giant cells, in addition to the polyblasts, fig. 5, Plate 11. These have been most numerous in cultures made from the hæmorrhagic core of certain types of tumour, from which the more common central necrosis was absent. These multinucleate giant cells are readily distinguishable from the carcinoma cells by their different nuclear structure, and the manner in which they segregate trypan blue.

In a few cultures in rat serum, after several days fibroblasts have grown out from the explants, spreading themselves on the glass. By so doing they appear to have provided a support for a certain number of carcinoma cells to wander out. Occasional tongue-like outgrowths of carcinoma cells also present the appearance of having been drawn out by the outgrowth of fibroblasts. They have not been seen to grow into sheets, but usually become detached from the glass, and shrink back to the explant.

Although a good sheet growth of carcinoma cells has not been obtained in rat serum, yet outgrowths have been obtained in fowl serum. The number of cultures out of twelve showing sheet growths of carcinoma cells in an experiment with different media was as follows :—

14.11.33. 63/187B

Mouse serum	Rat serum	Fowl serum
6	0	4

It is of interest to note that mouse and fowl sera are slightly more viscous and opalescent than rat serum.

6. *Mouse Carcinoma* 206

(*a*) *Growth in Mouse and Rat Sera*—This is the other tumour, the account of which in my former paper requires correction and amplification. Some tumours of this strain can be grown as sheets of carcinoma cells both in mouse serum and in rat serum. The best sheet growths have been obtained in mouse serum, although the carcinoma cells tend to contain more fat droplets than in rat serum. The percentage of sheet growth of carcinoma cells obtained varies very considerably from time to time. Some tumours have not given a single outgrowth of carcinoma cells in twenty-four cultures put up in rat serum Usually some sheet growth occurs in mouse serum, but negative results have been obtained. The difference between growth in rat serum and rat plasma is often very striking as in the following experiment :—

21.4.33. 206/599A

12 cultures in rat serum	No sheet growths of carcinoma cells, but large outgrowth of polyblasts with some fibroblasts.
6 cultures in rat plasma........	Extensive sheet growths of carcinoma cells with polyblasts.

Although the experimental conditions are kept as near constant as possible, yet when it is essential to employ such a complex medium as serum it is impossible to eliminate the possibility of variations in its composition when it must of necessity be obtained from different animals over a long period of experimentation. That differences in growth depend upon the state of the tumour, irrespective of variations in the medium, is seen when two tumours of different generations are put up at the same time in the same medium. The results of a recent experiment are given in Table II.

Table II—Number of Cultures out of Twelve showing Sheet Growth of Carcinoma Cells in the same Media

	206/613 A	206/616 A
In rat serum	4	0*
In mouse serum	5	11

* These cultures showed an extensive outgrowth of polyblasts.

The age of the rats from which the serum has been obtained has not been found to influence the outgrowth of carcinoma cells, but diluting the serum with distilled water appears to inhibit the growth of carcinoma cells. The results of a typical experiment are given in Table III.

Table III—9.5.33. 206/601A. Number of Cultures out of Twelve showing Sheet Growth of Carcinoma Cells

Serum from 7-weeks-old rat	Serum from old rat (2½ years)		
	Undiluted	2, serum : 1, water	2, water : 1, serum
10	9	4	0

The type of growth obtained from explants of carcinoma 206 varies considerably. Small fragments of some tumours grow as perfect sheets of beautifully spread carcinoma cells with very few or no polyblasts. Explants of other tumours give rise to a considerable growth of fibroblasts in addition to carcinoma cells and polyblasts. The fibroblasts frequently extend beyond the carcinoma cells as Drew (1923) described, but no indication has been found of fibroblasts bringing about differentiation of the malignant cells. In some cultures there are very few carcinoma cells but numerous fibroblasts, the growth of which can be maintained by subculturing. Fig. 6, Plate 12, shows such a culture, seven days after subculturing. Trypan blue was added to this twenty-four hours after explantation. The fibroblasts segregated the dye in the characteristic manner as fine droplets.

I have pointed out above that explants of some tumours give rise to outgrowths of polyblasts alone. This can occur in an alkaline medium, as the following experiment shows :—

12.4.33. 206/598A

Medium—Rat serum prepared six days previously, and kept in a cotton-wool-plugged tube in an ice safe. p_H 8·4. To some of this serum lactic acid was added giving p_H approx. 6·0.

Of 12 cultures in medium with p_H 8·4, eleven showed extensive outgrowths of polyblasts.

Of 12 cultures in medium containing lactic acid, p_H 6·0, none showed any growth.

Fig. 7, Plate 12, is a photomicrograph of a part of the extensive growth of polyblasts in a seven-days-old culture in alkaline serum, p_H 8·4.

(*b*) *Growth in Immune Rat Serum*—Since the malignant cells of mouse carcinoma 206 will, under favourable conditions, grow in the form of sheets of well-spread cells in rat serum, cultures of this tumour afford a favourable material for investigating whether the serum of animals resistant to the growth of transplantable tumours exercises any injurious action upon cancer cells. Experiments have therefore been carried out with the serum of rats resistant to the growth of the Jensen rat sarcoma. In the course of the routine transplantation of this tumour rats in which the tumour failed to grow were put aside. At subsequent routine transplantations these received further inoculations with minced tumour (usually 0·1 cc). At the same time as most inoculations were made, some of the same tumour was put up as tissue cultures. On each occasion this was done, growth of the malignant cells occurred *in vitro*. In none of the resistant rats did tumours grow at any time. Serum from these rats was prepared immediately before setting up the cultures. The design and results of an experiment are given in Table IV.

Table IV—22.11.33. 206/610A. Number of Cultures out of Twelve showing any Sheet Growth of Carcinoma Cells

	Mouse serum	Rat serum	Serum of resistant rat*
Undiluted	**11**	1	**5**
Diluted with ¼ Ringer solution	**12**	7	**8**

The bold figures denote more extensive growths.

* Dates on which resistant rat was inoculated with Jensen rat sarcoma: 21.6.33, 4.7.33, 18.7.33, 1.8.33, 15.8.33, 29.8.33, 7.9.33, 10.10.33, 24.10.33, 8.11.33.

Growth in this experiment was best in mouse serum, and rather better in the serum of the resistant rat than in that of a normal rat, but in a second experiment there was no difference between the growth in the two latter media.

Another type of experiment has been carried out and the results are given in Table V.

In this experiment the sera prepared on the first day of the experiment were used when the media were changed after the first two days growth, the media having been kept in the meantime in the ice safe. The criticism might therefore be raised against this experiment that any specific anti-malignant bodies present in the serum of the resistant rats might have lost their potency after two days. Another experiment was therefore performed. Cultures of the tumour were made in normal mouse serum. After 48 hours twelve of the best sheet growths were selected and the serum of six was renewed with some freshly prepared serum from a young rat, and of the other six with some freshly prepared serum from a resistant rat, which had received twelve inoculations with Jensen rat sarcoma. There was no appreciable difference between the subsequent growth of the twelve cultures. Fig. 8, Plate 13, shows numerous mitoses in a culture which had grown for two days in mouse serum, and for a further day in resistant rat serum.

Table V—28.11.33. 206/611A

Days				Number of cultures out of 6 showing good sheet growth of malignant cells
1	2	3	4	
Mouse serum		Mouse serum		6
		Resistant rat serum		4
Resistant rat serum*		Resistant rat serum		5
		Mouse serum		4

* Dates on which resistant rat was inoculated with Jensen rat sarcoma: 4.7.33, 18.7.33, 1.8.33, 15.8.33, 29.8.33, 7.9.33, 10.10.33, 24.10.33, 8.11.33.

Such experiments as these fail to give any insight into the immediate result of applying serum from a resistant animal to a healthy culture. It is conceivable that the serum may contain specific anti-cancer bodies in such a dilution that it could only act upon the well-spread cells at the extreme periphery of the growth, and these cells may ultimately recover. That this is not so has been determined by taking two-days-old cultures in which the carcinoma cells have grown as sheets in mouse serum, washing off the medium with Ringer solution, then adding a drop of freshly prepared serum from a resistant rat which had received twelve inoculations with Jensen rat sarcoma. Such cultures have been transferred to a warm box at 37° C, and kept under observation for two hours, both by transmitted light and by dark ground illumination. No injury to the carcinoma cells was observed. Examination by dark ground illumination is probably the best cytological method for detecting injury to cells. I have observed filamentous mitochondria in cultures to which very dilute solutions of slightly toxic substances have been introduced, break up into short rodlets, become granular and then vesicular in less than 5 minutes. Injury to the cell is also shown by an increased rate of movement of cell granules, followed by coagulation, which is readily distinguishable by a cloudy appearance of otherwise clear cytoplasm. The nucleus also readily shows coagulation. Bright light alone for a short time brings about these changes. However, after an hour in " immune " serum the well-spread carcinoma cells at the periphery of a sheet growth showed no changes, either nuclear or cytoplasmic. Figs. 9–11, Plate 13, show the typical rod-shaped mitochondria (*m*) in carcinoma cells which have been bathed for an hour with the serum of a resistant rat, which had received twelve inoculations with Jensen rat sarcoma.

I am led to conclude, therefore, that the mouse carcinoma 206 grows equally well in normal or " immune " rat serum. There is no evidence that the serum of a rat resistant to the growth of a transplantable tumour (JRS) contains anything inimical to the growth of mouse carcinoma 206.

7. *Other Transplantable Tumours of Mice*

In addition to the tumours so far described, attempts have been made to grow others in rat serum. These include transplantable tar carcinomata 2146 and 173, mammary carcinomata 27, 113, 91, Twort carcinoma, and the Bonne tar sarcoma. Some outgrowth of carcinoma cells has occurred from explants of carcinoma 27, but relatively very few cells have spread on the cover glass. A good sheet growth has not been obtained. With none of the other tumours was there any outgrowth of malignant cells.

Sufficient work has not yet been carried out with these tumours to draw definite conclusions as to their behaviour in rat serum. Even a tumour such as 206, which gives extensive sheet growths at times, will sometimes fail to show any outgrowth of carcinoma cells, so that it is inadvisable to draw conclusions as to the behaviour of any tumour until one has studied its growth over a considerable period of time.

8. *Jensen Rat Sarcoma*

(*a*) *Growth in Normal Rat Serum*—A cytological study of tissue cultures of this tumour has been made by Fell and Andrews (1927) who worked with both plasma and serum cultures. They figured good growths of sarcoma cells in serum and distinguished between the fibroblast-like cells, and the cells of the monocyte-macrophage type, which I have called polyblasts in this communication.

The best sheet growths of this sarcoma are obtained when there are relatively few polyblasts, or after these cells have been eliminated by transferring the explants to fresh cover glasses. Thus in an experiment the following results given in Table VI were obtained.

Table VI—28.3.33. JRS/415A

Explant transferred to new covers on the second and fourth day	Serum renewed on the second and fourth day
Nine cultures out of 10 showed good sheet growth of sarcoma cells on sixth day. Very few polyblasts.	Eight cultures showed extensive outgrowth of polyblasts, and some scattered sarcoma cells on sixth day.

It is not necessary that the explant should be transferred to a fresh cover glass to obtain sheet growths of the sarcoma cells, for excellent growths have been obtained by moving an explant to another part of the same cover slip away from the outgrowth of polyblasts. Fig. 12, Plate 14, shows the growth obtained from an explant, which had been moved in this manner, after three days in rat serum. The culture was fixed two days after moving the explant away from the original outgrowth of polyblasts, part of which, with scattered sarcoma cells, is shown in fig. 13, Plate 14. Growths of this sarcoma have also been obtained in mouse serum, but the sarcoma cells then usually contain more fat droplets than do the cells of the same tumour grown in rat serum.

(*b*) *Growth in the Serum of Resistant Rats*—Serum from rats resistant to Jensen rat sarcoma, which had received repeated injections of tumour emulsion as described on p. 285, has also been used to grow this tumour *in vitro*. A typical experiment is given in Table VII.

Table VII—18.7.33. JRS/419A and Fibroblasts

	Serum from tumour-bearing rat (Autologous)	Serum from normal rat (Homologous)	Serum from resistant rat (" Immune ")
Number of cultures out of 12 of JRS showing growth of sarcoma cells	10	11	10
Number of cultures out of 12 of rat embryo heart showing growth of fibroblasts	8	10	12

Seven such experiments have been performed. In general the growth obtained in "immune" and homologous serum has been very similar, although on the whole slightly better in "immune" serum. Growth in autologous serum has not usually been quite so good. Nothing analogous to Woglom's (1933) results *in vivo* with Crocker rat sarcoma 39 has been obtained.

9. *Other Rat Sarcomata*

Two other rat sarcomata, Nos. 41 and 46 of this Laboratory, have also been grown in rat serum, and have given good sheet growths of sarcoma cells.

Rat sarcoma 46 was also grown in the serum of rats resistant to JRS. The sarcoma cells grew rather better in the "immune" serum than in the serum of normal rats.

10. *Discussion*

(*a*) *The Nature of the Growth in Fluid Media*—The experiments which have been described have shown that the different strains of mouse tumours exhibit specific variations when grown in mouse and rat sera. By the technique we have employed some strains of tumours have grown as extensive sheets of malignant cells in both sera; others in neither; while there are tumours which have given extensive sheet growths in mouse serum and not in rat serum. It is interesting to observe that polyblasts and frequently fibroblasts grow out from explants of all tumours in both sera. The carcinoma 63 has been grown in sheet form in both mouse and fowl sera, only very rarely has there been a tendency to sheet growth in rat serum. The latter medium can even bring about the complete destruction of sheets of malignant cells, leaving polyblasts uninjured. As I have already pointed out this does not necessarily mean that rat serum is toxic to the carcinoma cells of this tumour, because malignant cells of explants put up in rat serum will grow out when transferred to mouse serum. Also I showed in my previous paper that carcinoma cells within the explant undergo active division in rat serum. Whether cells will migrate from the explant or not in a fluid medium depends probably upon the capacity of the cells to adhere to the cover glass in that particular medium, rather than upon the growth inhibiting or stimulating properties of the medium. A toxic substance may destroy all the cells both of the outgrowth and of the explant; but a medium which will permit of cell growth may yet prevent any outgrowth on the cover glass. Sheet formation has been obtained *in vitro* which presents the appearance of having arisen as the result of cells migrating from the explant, without any evidence of division having occurred. Thus in some cultures of the skin of the salamander larva extensive growths of epidermal cells were obtained; but no cell division was observed, either before or after fixation.

The variations in the type of outgrowth obtained with any one strain of tumour when it is cultured over a long period presents fresh problems. The number of polyblasts migrating from a tumour explant depends principally upon the extent to which the animal in which the tumour grew had opposed resistance to its growth. In my previous paper I pointed out that a very extensive growth of polyblasts is obtained from explants of tumours the growth of which had begun to be arrested *in vivo*. A young vigorously growing tumour when grown *in vitro* usually gives rise to very small outgrowths of polyblasts, occasionally none at all. It is such tumours which produce the best sheets of malignant cells when grown in a suitable medium.

It is highly probable that variations in growth also depend upon differences in the serum from different animals. Until a suitable synthetic medium can be prepared this aspect of the problem remains beyond an experimental analysis.

(*b*) *Variations in the State of Activity of Malignant Cells and Growth in Fluid Media*—Besides the extrinsic factors which influence the growth of malignant cells *in vitro*, our experiments indicate that there are other factors intrinsic to the cells. The technique employed is exonerated from entire responsibility for differences in growth occurring from time to time by the fact that two tumours of the same strain but different generations behave differently when put up in the same medium at the same time. We do not know what cellular changes are responsible for these fluctuations in growth, but since they also influence the adhesion of the cells to a glass surface they probably involve alterations in the colloidal state of the cells, or at least of the cell membranes. As yet we have not been able to control these changes experimentally, and are therefore unable to say how any tumour may behave in fluid media under special conditions, so it is at present inadvisable to make absolute statements about the growth of any tumour in serum. There may be some phase in the growth of all tumour cells when they will grow in sheet form in serum. If so, we have never explanted sarcoma 37, or the Crocker sarcoma at this critical stage.

Apart, however, from how tumours may behave on some rare occasions when one assumes they are in a peculiar phase of activity, it is possible to speak of the general type of their growth in serum. Thus one can be reasonably certain of being able to obtain an outgrowth of malignant cells of mouse sarcoma 2529 in rat serum, but not of sarcoma 37; also of mouse carcinoma 206, but not of carcinoma 63. There is no doubt about the marked differences in the general behaviour of the different strains of tumours when explanted in mouse and rat sera.

(*c*) *The Reaction of Malignant Cells to Trypan Blue*—In my former paper I suggested that the failure of mouse cancer cells to wander out on a glass surface might be due to an increase in the fat or lipin content of their plasma membranes, which would also offer an explanation of their inability to segregate a water soluble dye, such as trypan blue. It is of interest to note in this connection that the malignant cells of sarcoma 37 and the Crocker sarcoma, which have not been grown in sheet form in either rat or mouse sera, do not segregate trypan blue as do fibroblasts *in vivo* or *in vitro*. On the contrary, the malignant cells of sarcoma 2529 are not stained *in vivo* by injections of trypan blue sufficient to stain intensely fibroblasts and polyblasts of the stroma, yet these sarcoma cells grow as sheets both in rat and mouse sera, and can be vitally stained by the addition of trypan blue to the cultures. The cells of the rat sarcomata that have been studied present similar peculiarities (Ludford, 1932, *b*). When cells spead themselves out in tissue cultures their surface areas are considerably increased. The increase is greatest with the widely spread cells around the periphery of a sheet growth in serum. Such cells are frequently reduced to little more than a film of protoplasm adherent to the cover glass, which is apparently under a tension, because injurious agents acting upon such cells cause them to contract and round off; even a brilliant light is sufficient to bring about this shrinkage. Assuming therefore that with well-spread cells the plasma membrane is stretched, alteration in its ultramicroscopic structure is highly probable. When such cells are bathed with a dye solution maintained at a higher concentration than is possible *in vivo*, dyestuff may be able to penetrate although unable to do so under the conditions obtaining *in vivo*. The fact that some malignant cells are able to spread out on a glass surface in serum, and not others, would have to be attributed to differences in the colloidal state of the protoplasm of the various strains of tumours.

In support of this conception it is worthy of note that when well-spread tumour cells growing in plasma cultures to which trypan blue has been added are examined under dark ground illumination, dye droplets can often be observed in their cytoplasm although these escape detection when examined by transmitted light. This applies to the spread cells of most of the strains of tumours, even those such as the Crocker sarcoma, which under more normal conditions of growth do not segregate acid dyes.

(*d*) *The Relation between Malignant Cells and Polyblasts*—That there is some relation between cells of the macrophage type and sheet growth of malignant cells has been indicated by several of our experiments. The conditions under which we have obtained sheet growths of malignant cells in suitable fluid media have been :—

(1) when the explant contains no polyblasts, or very few;

(2) when the polyblasts have wandered well out into the medium away from the explant;

(3) when the polyblasts have been filled with fat droplets, or become rounded and apparently inactive;

(4) when the polyblasts have been eliminated by subculturing.

Certain strains of tumours, which I have already mentioned, have not yet been grown as sheets of cells under any of these conditions.

No evidence has been found that the polyblasts produce substances injurious to the malignant cells; their action seems rather to be a mechanical one.

Unlike the conditions existing in plasma cultures where cells can migrate from the explant on the fibrin reticulum, in serum cultures the cells have only the surface of the cover slip for their support. Hence a large outgrowth of active polyblasts may tend to crowd out the malignant cells. Then the polyblasts are actively phagocytic. In living culture they have been seen to clasp the processes of malignant cells causing them to retract. By their phagocytic efforts they appear to offer resistance to the attempts of the malignant cells to move along the surface of the cover glass.

(*e*) *Immunity to the Growth of Transplantable Tumours*—So far as my previous criticism of Lumsden's (1931) work on immunity *in vitro* was based on my erroneous conclusion that "in serum cultures only non-malignant cells wander out from the explants," I unreservedly withdraw it. One of his photomicrographs shows that he had obtained emigration of malignant cells (Lumsden, 1931, fig. 7), but the culture illustrated was not shown treated with "immune serum." The crucial point of my criticism of his work was, and remains, that he failed to distinguish between malignant cells and cells of the monocyte-macrophage type. In the experiments which he performed with mouse carcinoma 63, his photomicrographs show that he mistook the cells of the monocyte-macrophage series for carcinoma cells. This is clearly evident, as I pointed out in my former paper, in "figs. 9, 11, 18, 24, and 25 of tumour 63 in the recent review of his work, in the 'Amer. J. Cancer,' 1931" (Ludford, 1933, p. 259). He frequently employed rat serum as a culture medium for this tumour, failing to recognize, as described in this communication, that apart from very exceptional circumstances, only cells of the monocyte-macrophage type migrate from the explants in this serum. His figures do not show malignant cells. I pointed out in my previous paper that any substances for which a specific destructive action on cancer cells *in vitro*, is claimed, as Lumsden has done (1931, p. 590), should leave the cells of the monocyte-macrophage type uninjured, while destroying the malignant cells. Lumsden's photomicrographs (1931) show that his "immune sera" destroy the non-malignant cells, but fail to reveal the fate of the malignant cells. My identification of the cells of the monocyte-macrophage type is based upon the following considerations.

(i) Their morphology: relatively small nuclei, vacuolated cytoplasm, irregular shape and outline: and their active movement.

(ii) Their characteristic appearance by dark-ground illumination: relatively large vacuoles and granules near the nucleus, and clear peripheral cytoplasm with rod-shaped mitochondria.

(iii) Their intense staining with trypan blue, which renders them readily distinguishable from malignant cells even in those strains of tumours, the malignant cells of which show some vital staining.

(iv) The vital staining of their cytoplasmic vacuoles with basic dyes such as neutral red. The coloured vacuoles characteristically form a group at the side of the nucleus (Lewis, 1927).

(v) The same kind of cells migrate from the explants of all types of tumours, sarcomata and carcinomata. They can be most easily distinguished from the malignant cells when the latter are exceptionally large (*e.g.*, carcinoma 27), or when the malignant cells contain well-defined inclusions as in carcinoma 27, Passey's melanotic sarcoma, or Foulds' mast cell sarcoma.

(vi) With suitable tumour explants it is possible by repeated transference to fresh cover glasses and fresh medium to eliminate the non-malignant cells, and obtain a relatively pure culture of the malignant cells (p. 280), see also Carrel and Ebeling (1928).

(vii) The same type of cells migrate from the explants of non-malignant tissues, especially in cultures of lymph node, and embryonic skin.

I contend that so far as one is able to judge from Lumsden's photomicrographs he did not investigate the action of his immune sera on cultures of vigorously growing malignant cells. It therefore seemed desirable to reinvestigate this aspect of the problem. My experiments with the Jensen rat sarcoma completely confirm his conclusion that, "if the serum of a rat immunized against JRS is applied to cultures of rat-grown JRS cells, it does not injure them . . ." (Lumsden, 1931, p. 593). In the same paragraph he states that the serum of a rat rendered "hyper-immune" to Jensen rat sarcoma contains specifically anti-malignant bodies which have "markedly deleterious effects" upon mouse-grown M.63 cells. In my experiments with this tumour it was found that the sheets of malignant cells grown in mouse serum could often be destroyed by the addition of the serum of a young normal rat, and I have suggested that this result is due to the adhesion of the malignant cells to the surface of the cover glass being altered by rat serum. It was necessary, therefore, to experiment with a tumour the malignant cells of which would grow well in sheet form in normal rat serum. Such a tumour is the mouse carcinoma 206. When the serum of a rat "hyper-immune to JRS" was applied to healthy cultures of this tumour grown in mouse serum, no deleterious effects, either immediate or late, were observed (p. 288).

If the serum of an animal resistant to tumour growth contains nothing inimical to the growth of malignant cells, how then are we to explain the failure of a tumour graft to grow in a resistant animal? I would suggest tentatively that it is the cells of the monocyte-macrophage series which are principally responsible. Murphy (1926) has previously expressed similar views. Around a tumour graft in a resistant animal they collect in large numbers (Mottram and Russ, 1917) forming a barrier depriving the malignant cells of adequate nutrition, and preventing fibroblasts and capillaries from growing in and forming a stroma (*see* Russell, 1908; Woglom, 1912). By their phagocytic activities they interfere with the malignant cells, attempt to throw out protoplasmic processes in their search for a support on which to wander out into the surrounding tissues. Thus the malignant cells are segregated so that they slowly die, unless the macrophage barrier is broken down or these cells are inactivated as in my experiments with acid dyes and metallic colloids (Ludford, 1932, *a*).

11. *Summary*

(1) By the technique we have employed significant differences have been found in the behaviour of different strains of tumours in mouse and rat sera, figs. 3 and 4, Plate 11. Some tumours have not been grown as sheets of malignant cells in either mouse or rat sera; other tumours have given good sheet growths in mouse serum, but not in rat serum; while still others have grown in both sera.

(2) Tumours of the same strain, but different generations, exhibit considerable variations in the extent and the type of their growth, even when explanted at the same time and in the same medium. Their behaviour *in vitro* confirms observations made *in vivo* that fluctuations occur from time to time in the biological state of malignant cells.

(3) Fibroblasts and cells of the monocyte-macrophage series of all the tumours studied grew well in both rat and mouse sera, figs. 6 and 7, Plate 12.

(4) The cells of tumour strains which have not been grown in sheet form in rat serum, grow as such in rat plasma.

(5) Explants of mouse tumour 63 which have given rise to no outgrowths of malignant cells after four days in rat serum have given sheet growths 24 hours after being transferred to mouse serum. Controls kept in rat serum have still remained quiescent.

(6) Sheet growths of carcinoma 63 in mouse serum have been broken up by washing the cultures and adding rat serum. Fresh sheet growths have arisen on transferring again to mouse serum. Control cultures treated in the same way but kept in mouse serum all the time have continued their growth.

(7) It is suggested that whether, or not, cells form sheets from explants in a fluid medium depends upon the adhesion of the cells to glass in that particular medium rather than upon the growth-promoting or growth-inhibiting properties of the medium. Cells which fail to migrate from the explant may yet undergo mitosis within the explant.

(8) The malignant cells of mouse carcinoma 206, which in certain states of the tumour form sheets in rat serum, grow equally well whether the serum is obtained from a young rat, or from a rat " hyperimmunized " against Jensen's rat sarcoma, fig. 8, Plate 13. No evidence has been found that such "immune" serum contains anything inimical to the growth of this tumour *in vitro*, figs. 9–11, Plate 13.

(9) Certain differences in the reaction of malignant cells to trypan blue *in vivo* and *in vitro* are attributed to the maintenance of a higher concentration of dye, and the spread out condition of the cells in tissue cultures.

(10) The presence of large numbers of active cells of the macrophage type has been found to interfere with the sheet formation by malignant cells in fluid media, figs. 1 and 2, Plate 10. This is regarded as due partly to crowding out of the malignant cell on the surface of the cover glass, and partly to the phagocytic activities of the polyblast offering resistance to the movement of the malignant cells along the surface of the coverslip. It is suggested that it may be the activity of cells of this type accumulated around a tumour graft in an " immune " animal which prevents its growth.

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DESCRIPTION OF PLATES

Abbreviations used :—B., trypan blue ; Im., immune ; M., mouse ; R., rat ; S., serum.

An oblique stroke, thus, /, implies that the explant was transferred to a fresh cover glass ; a horizontal stroke, thus, —, implies that the medium of the culture was renewed.

Figures denote the number of days the explant remained in the medium, *e.g.*, RS1/2/3 means a culture was put up in rat serum, the explant was transferred to a fresh cover glass after one day, again after another two days, and remained three days in the last medium. MS 1—2—3 B2 means a culture was put up in mouse serum, and the medium was renewed after one day, again after a further two days, and stayed in the last drop of medium for three days, when a small drop of 0·5% trypan blue was added, and remained for two days.

PLATE 10—Mouse Sarcoma 2529

FIG. 1—Culture in rat serum showing the outgrowth of well-spread sarcoma cells. Polyblasts have been eliminated by transference of the explant to fresh covers. RS 1/2/3. (× 60.)

FIG. 2—Culture in rat serum, showing part of the extensive outgrowth of polyblasts, and a few scattered sarcoma cells, (S) spread on the cover glass. RS 1—2—3. (× 60.)

PLATE 11—Mouse Carcinoma 63

FIG. 3—Explants in rat serum showing a tendency to sheet formation. This is the best outgrowth of carcinoma cells, which has been obtained in rat serum. RS 2—3. (× 17.)

FIG. 4—Culture of the same tumour as fig. 3, put up at the same time in mouse serum. Extensive outgrowth of carcinoma cells. MS 2—3. (× 17.)

FIG. 5—Culture of another tumour in rat serum showing the rounded explant and outgrowth of polyblasts, macrophages, and multinucleate giant cells. RS 1—1 B2. (× 83.)

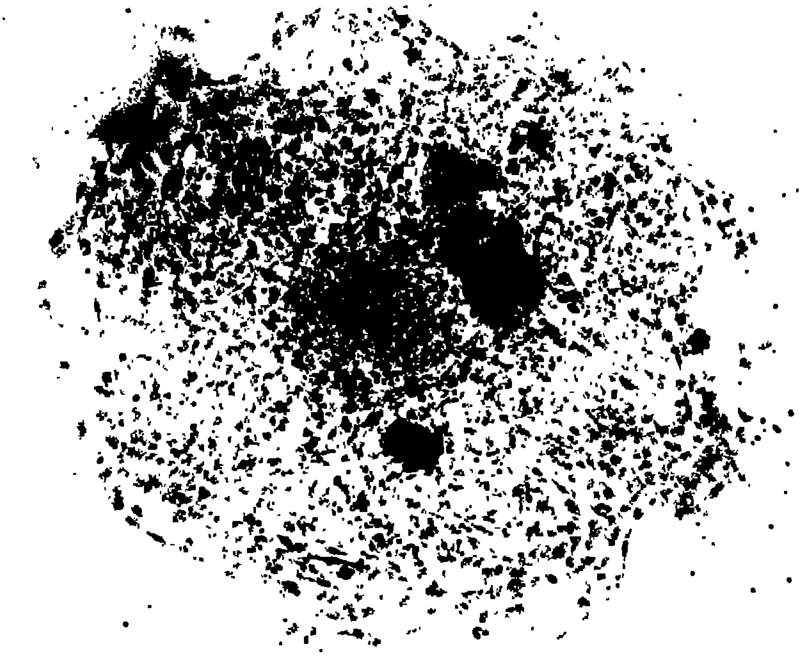
PLATE 12

FIG. 6—Outgrowth of fibroblasts from an explant of mouse carcinoma 206. RS 1 B2/RS 7. (× 62.)

FIG. 7—Part of an extensive outgrowth of polyblasts and macrophages from an explant of mouse carcinoma 206. RS (p_H 8·4) 7. (× 62.)

PLATE 13—Mouse Carcinoma 206, in Serum of Rat "hyperimmune" to Jensen's Rat Sarcoma.

FIG. 8—Mitosis of carcinoma cells 24 hours after the addition of "hyperimmune" (12 negative inoculations of JRS) rat serum. MS2—1mRS1. (× 333.)



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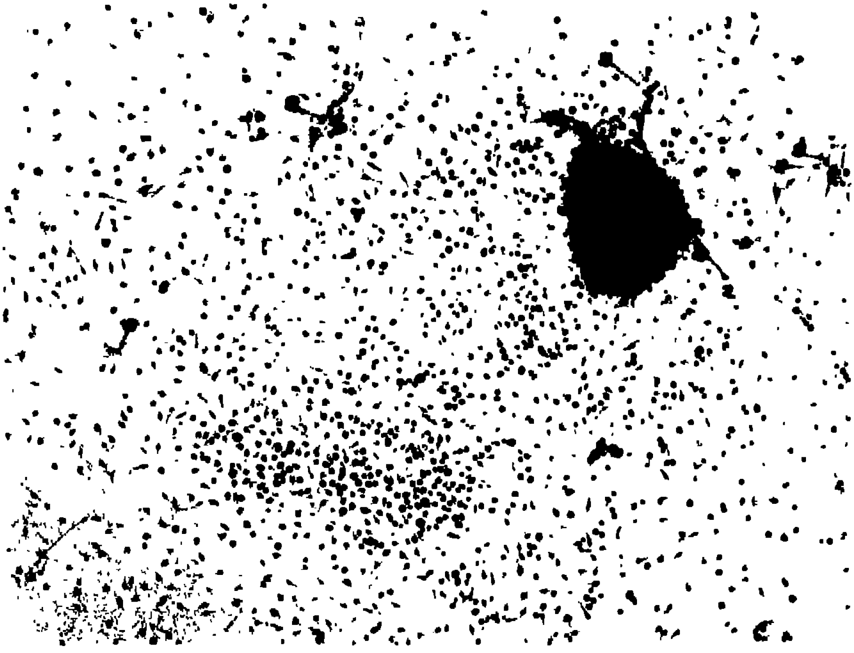

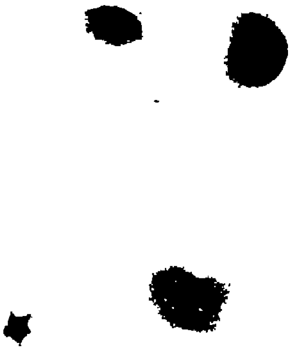

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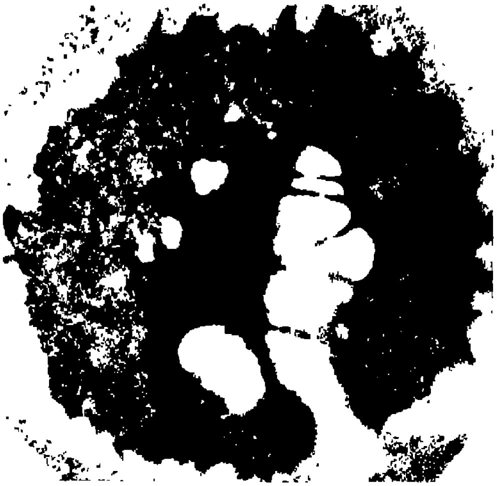

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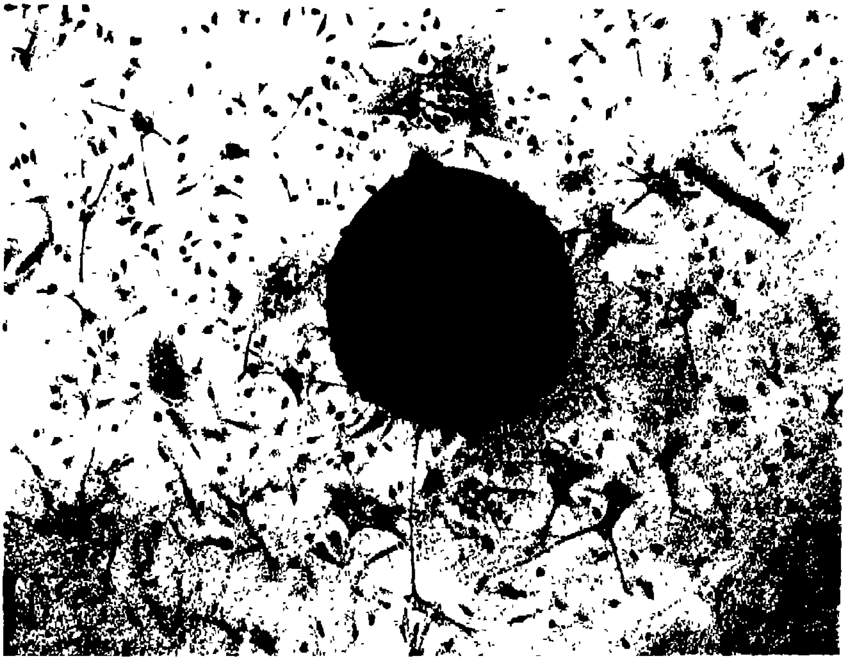

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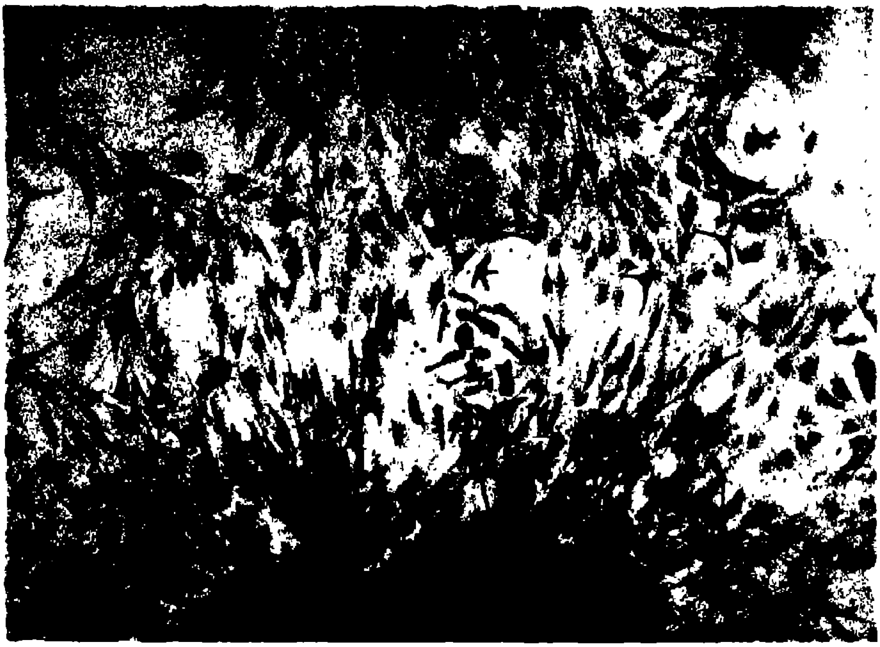

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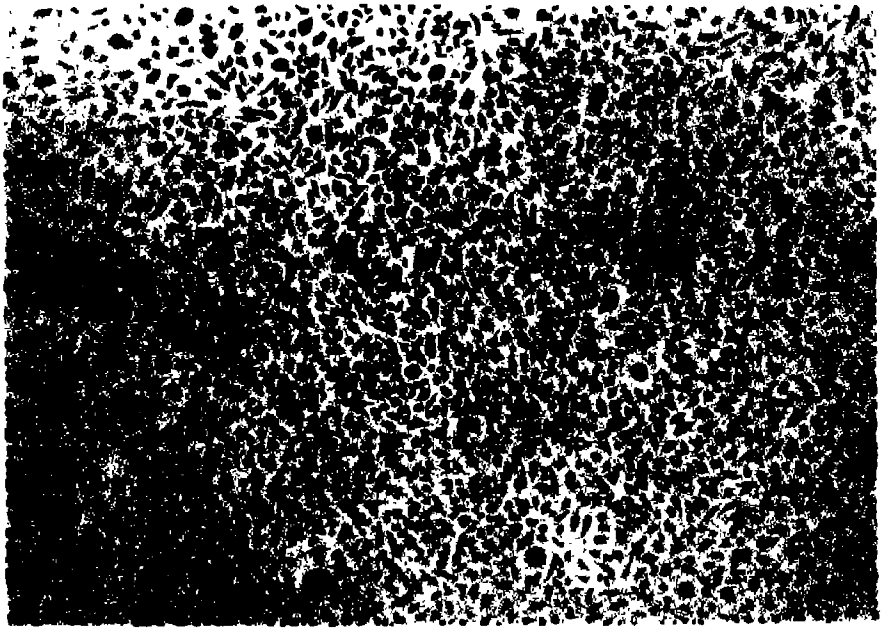

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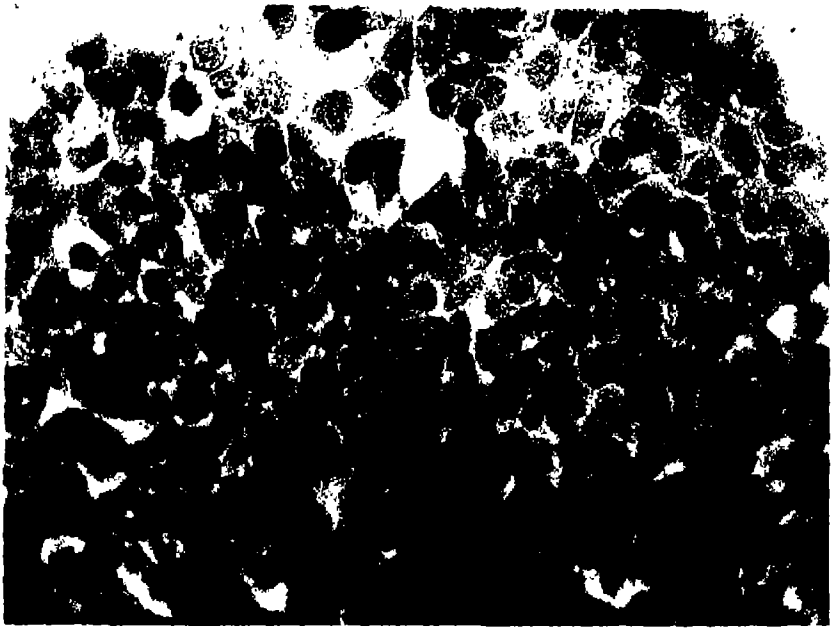

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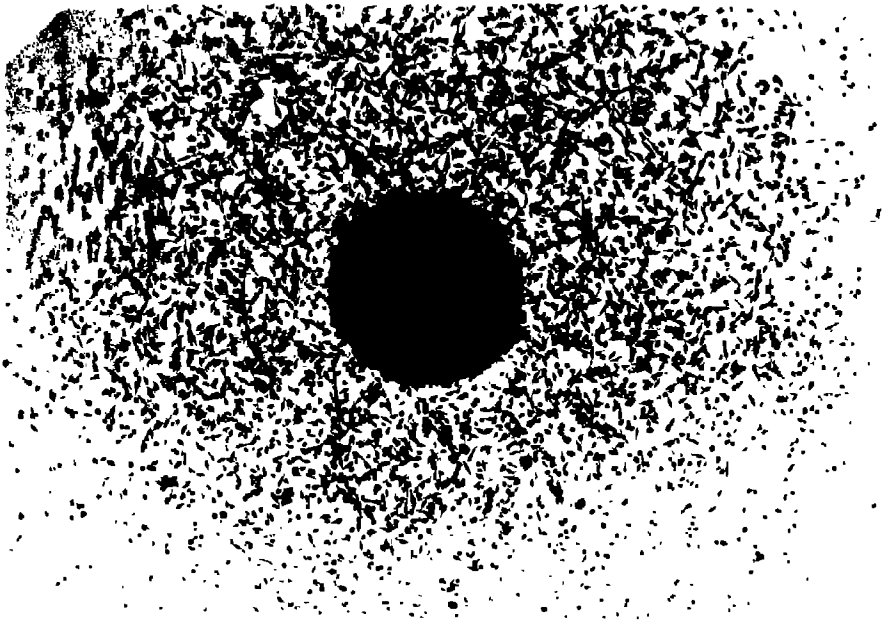

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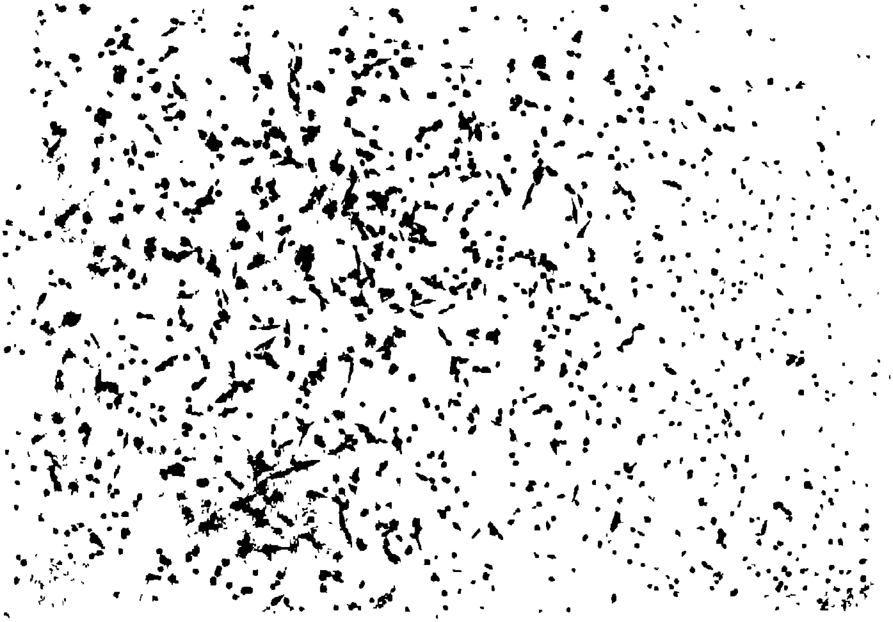

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FIGS. 9–11—*Peripheral cytoplasm of carcinoma cells on the margin of a culture, grown* in mouse serum for two days. This serum had been removed, and the culture washed with two changes of Ringer solution, and a drop of freshly prepared serum added from a resistant rat which had received 12 inoculations with Jensen's rat sarcoma. The figures show the unaltered filamentous mitochondria after one hour in the "hyper-immune serum." *m.*, mitochondria; *n.*, nucleus. MS2—Im RS 1 hr. (× 1500.)

PLATE 14—Jensen's Rat Sarcoma

FIG. 12—Outgrowth consisting mostly of sarcoma cells from an explant which had been moved on the third day away from the original extensive outgrowth of polyblasts. $\mathrm{RS1{—}2{—}}\!\begin{array}{l}\rceil\\ 2\end{array}$ (× 41.)

FIG. 13—Part of the original extensive outgrowth of polyblasts with scattered sarcoma cells in the same culture as fig. 12. RS 1—2—2. (× 41.)

612 . 275

The Limit of High Flying when Breathing Oxygen

By Sir Leonard Hill, F.R.S.

(Received February 5, 1934)

The experiments herein described were carried out at the works of Messrs. Siebe Gorman, Ltd., with the co-operation of Sir Robert Davis, who kindly placed at the author's use the necessary apparatus. A small cylindrical copper chamber was used about 20 inches long and 12 inches wide. One end was hermetically closed, and the other fitted with a removable lid; a thick glass disc formed the larger part of this lid, and served as an observation window. An air-tight junction was made between the lid and the chamber by means of thick rubber washers lubricated with glycerine. When a positive pressure was used a set of bolts and nuts secured the lid to the chamber and made the closure air-tight. The chamber was made to withstand pressure up to +130 lbs. It was fitted with valved inlet and outlet tubes; one tube connected the chamber with a pressure gauge. For evacuating the chamber and studying the effect of low pressures, an efficient pump was used with the piston working in oil. The stroke of the pump was driven by a band off the workshop shafting. The chamber was evacuated by this means down to the lowest pressure required (70 mm Hg), in about 6 minutes. To indicate the pressure during evacuation the chamber was connected to the top of a barometer tube, which was fitted with a scale; the bottom of this tube dipped in a cup of mercury. The chamber was placed in the vertical position, and a wooden platform arranged inside on which the animals rested in full view under the glass window. The opening of the inlet tube used for ventilating the chamber was carried below this platform, so that the chamber could be thoroughly washed out with oxygen, or a mixture of oxygen and nitrogen. After the animals, *e.g.*, two rats, had been introduced, and the chamber closed, the inlet tube was connected, in the first series of experiments, with a cylinder of oxygen (99%) and the chamber well washed out with this gas. It was then repeatedly evacuated down to about 150 mm Hg, oxygen being admitted each time. This procedure secured a purity of oxygen in the chamber, as was shown by analysis. Finally the chamber was evacuated down to the pressure whereat symptoms of acute dyspnœa became manifest, a slow inflow of oxygen being maintained during this evacuation. The animals settle down, and quietly rest on the platform during the final evacuation, that is until the pressure drops to about 100 mm Hg, they then become restless, and when the pressure drops to 90–80 mm Hg their breathing becomes very deep and slow and they show other signs of want of oxygen. Rats make convulsive leaps towards the window of the chamber, and on coming to rest their limbs straddle out, guinea pigs and pigeons fall over, monkeys close their eyes and fall asleep when the pressure falls below 120–110 mm Hg, and then as the pressure falls below 100 sink lower and lower down, but are still able to balance themselves in the sitting posture. Below 90 mm Hg the head falls against the side of the chamber and the monkey is unable to recover its balance when the chamber is shaken. The animals quickly recover when oxygen is let in, thus the evacuation could be repeated several times with like result. Goats were observed in a larger chamber which took about 35 minutes to evacuate. They were at first in the standing posture, but lay down when the pressure fell to 100–90 mm Hg and made convulsive movements when it reached 90–80 mm Hg.

The conclusion was reached that about 85 mm Hg is the pressure at which severe symptoms occur for all these classes of animals. These experiments confirm the classical ones of Paul Bert (1878). He noted that a sparrow fell over when the pressure of oxygen fell to 75 mm Hg.

When breathing oxygen Jongbloed (1929) had exposed himself and another to 124 mm, while Talenti found 115 mm Hg the limit for full conscious control in the case of men. The author had the opportunity of testing this on Mr. Eric Taylor with the co-operation of Professor Barcroft (Barcroft and others,. 1931) and Wing Commander Marshall (1933). Mr. Taylor breathed oxygen from a Salvus apparatus, while the chamber (in this case containing air) was evacuated. He took notes in full view of the observation window.

The reduction of pressure proceeded without incident up to 35,000 feet, when slight loss of the subject's original fresh colour was noticed.

At 37,000 feet he counted his own pulse and wrote the result on paper, as shown in fig. 1, without any difficulty.

At 40,000 his colour was definitely paler than normal and Professor Barcroft remarked on this. At this height, the rate of climb was certainly not more than 1,500 feet per minute. The subject nodded when asked if he was quite comfortable and, objectively, appeared to be in no distress at all, though occasionally he took a deep, sighing respiration. Apart from this, his respiration was slightly hurried and very noticeably deeper than normal and he was seen to manipulate the oxygen apparatus so as to ensure an adequate supply of oxygen. He says that he was beginning about this time to be conscious of oxygen shortage which manifested itself in some difficulty of vision and hearing, requiring a definite mental effort to enable him to focus an object clearly.

Just below 40,000, he appeared less at ease and his colour less good. At 43,660 feet (114 mm Hg) he was seen to make an effort to write on a block of paper and the moment he began to do this he passed into a state of jactitating movement whereby every movement attempted became a series of short, rapid purposive jerks. The tablet fell from his knee and the pencil out of his hand, and it was difficult to determine whether the movements that he made after this were mere jactitations or attempts to recover the articles that he had dropped. All the movements were directed towards a point on the floor beside his right foot. His face had become an ashy-grey. He was visibly sweating and it was obvious that the experiment must be terminated immediately.

Air was then admitted to the chamber so as to bring him down at great speed to 42,000 feet where the pressure was held for a minute or two in the hope that he would recover. He did recover to the extent of sitting up, picking up paper and pencil and beginning to write; this writing is shown in the figure beginning with the word "slight." The first word was written with apparent ease and confusion only began with the repeated vertical strokes of the word "dimming."

As the apparent recovery seemed in danger of being lost, it was decided to abandon the experiment and the "height" was reduced at a fairly constant rate of about 3000 feet per minute.

The writing of the four lines beginning with the word "slight" occupied fully 2 minutes and at about 30,000 to 32,000 feet he stood up and showed this writing at the porthole.

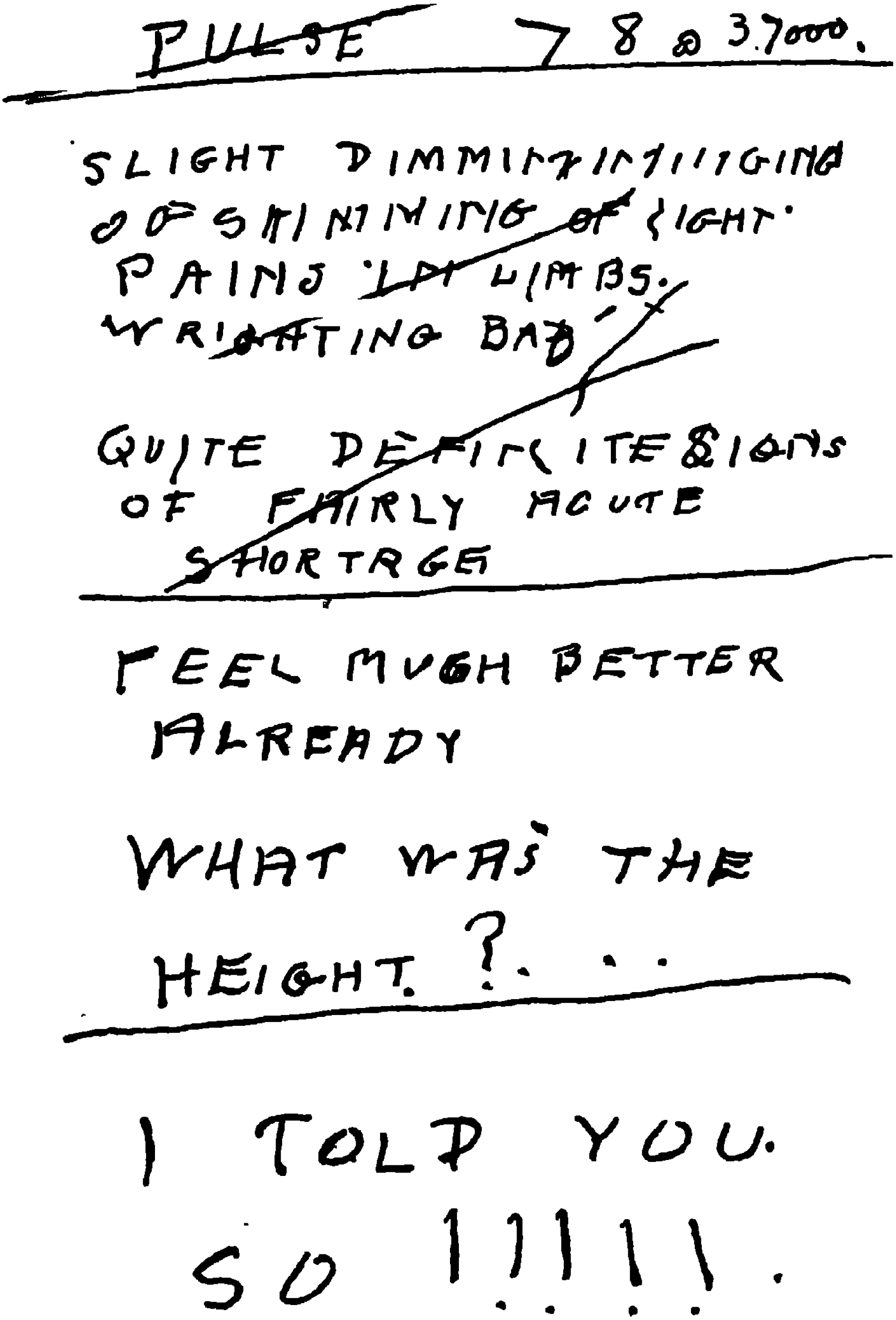
10,000 feet lower he wrote the three lines beginning with the word "quite," and this time his writing was much faster, probably half as fast as normal. By this time his colour had almost entirely returned and he appeared alert and once more to be taking an interest in the experiment.

On being told at his request the maximum "height" attained, he wrote the words "I told you so ! ! ! ! !" (referring to the opinion of one of those present that this height would constitute the limit of toleration) and displayed the high merriment that one associates with drunkenness or the administration of nitrous oxide. He says that this merriment was occasioned by the peculiar appearance of his earlier handwriting.

The remainder of the descent was uneventful.

On being released from the chamber, he climbed out easily and stood up without swaying.

His colour was red enough, but still with a slightly ashen tinge, especially about the lips and lobes of the ears.



PULSE 78 @ 37000.

SLIGHT DIMMINTINIIIGING OF SHINNINING ~~OF~~ LIGHT.
PAINS ~~LN~~ LIMBS.
WRI~~AT~~TING BAD

QUITE DEFINITE SIGNS OF FAIRLY ACUTE SHORTAGE

FEEL MUGH BETTER ALREADY

WHAT WAS THE HEIGHT ? . . .

I TOLD YOU. SO !!!!! .

Fig. 1

On being questioned he had evidently no knowledge of having been unconscious, though all who had seen him were agreed that he had undoubtedly been so. He had no recollection of dropping the pad or pencil or of picking either of them up. When writing the paragraph beginning "slight," he was aware of the need for concentration and that he was writing badly, but not of the repetitive nature of this writing. He said that he had felt no pain beyond some intestinal distension and some ache in the muscles of the front of the arms which disappeared before the pressure had returned to normal. It is most important to note that this subject states that at the moment when he was, in fact, fainting, he felt that although he was slightly embarrassed by oxygen-want, he could have gone on to a much greater height. This corresponds with the same subject's feelings on a previous occasion when he fainted from oxygen-want.

Previous to this experiment Captain C. F. Uwins flew successfully to a height of 44,000 feet, where the pressure fell to 115 mm Hg. To go higher it is necessary to enclose the pilot in a special dress wherein the pressure can be made higher than the atmospheric pressure. Acting on the advice of Dr. J. S. Haldane (1922), Sir Robert Davis has contrived such a dress, and enclosed in this and breathing oxygen, from an oxygen-breathing apparatus, Mr. Mark Ridge was successfully submitted in a chamber to an evacuation which corresponded to a height of 90,000 feet. By this simple means the possible height of flying has been doubled; there is no need for expensive and heavy observation chambers used at ordinary air pressure by Piccard, and others, for their balloon observations at great heights.

Having determined the possible limit when breathing oxygen, a second series of experiments was carried out, which enabled the author to measure the combined tension of water vapour and carbon dioxide in the alveolar air, and so find the actual tension of oxygen at the point when acute signs of dyspnœa occur. To effect this, a cylinder containing a compressed mixture of oxygen 5% and nitrogen 95% was connected with the inlet of the chamber, and the pressure raised rapidly to a point which gave a sufficient partial pressure of oxygen, *e.g.*, +3 atm. The chamber, while at this pressure, was then very thoroughly ventilated with the mixture. To effect more complete washing out, the pressure was next raised to +6 atm and lowered to +3 atm several times. When a mixture of 5% of oxygen and 95% of nitrogen is breathed at a pressure of three or four atmosphères (abs.) the partial pressure of oxygen is equal to 20% of one atmosphere. On now lowering the pressure down to one atmosphere (abs.) the partial pressure of oxygen fell to 5% of one atmosphere and the animals breathed deeply and showed signs of acute dyspnœa. Using this mixture containing only 5% of oxygen, it is clear that the carbon dioxide and water vapour in the alveoli have a negligible effect on the alveolar oxygen partial pressure. Hence we can take the external oxygen partial pressure as being equal to the oxygen pressure in the alveoli. This comes to about 7·6 × 5 = 38 mm Hg. When mixtures containing 2½ or 2% of oxygen were used the same partial pressure of oxygen was found to be critical.

Now we have seen that when almost pure oxygen was breathed and the chamber evacuated, symptoms of dyspnœa occurred when the pressure fell to about 85 mm Hg. Taking 38 from 85 we have 47 left as the combined alveolar partial pressure of water vapour and carbon dioxide. A further series of experiments showed that when this figure 47 was taken the oxygen partial pressure came out in good agreement with the figure 38, whatever the gas mixture which was used. The results are shown in Table I.

Christie and Loomis (1932) by voluntary rapid shallow breathing lowered, in man, the alveolar water vapour tension to 38 mm Hg and the carbon dioxide tension to 22 mm Hg, a total of 60 mm Hg.

Campbell (1927–1930) found in animals living in a partial pressure of oxygen equal to 7·5% of one atmosphere a tension of carbon dioxide in the tissues of about 20 mm Hg. In the above experiments animals (before showing signs of acute dyspnœa) breathe slowly and deeply ; this breathing must help to wash out the carbon dioxide and the output of this gas must also be lowered through oxygen-want.

Jongbloed (1929) found an alveolar tension of carbon dioxide of 27–30 mm Hg in three men, and 20·2 in one, when exposed to a pressure of 139–124 mm Hg, while breathing oxygen.

Everest climbers at the great heights suffer much from dry throats ; the panting respiration and the very dry atmosphere together must tend to lower the alveolar water vapour tension. Their body temperature may fall, the temperature of the expired air being lower than at sea-level. By such means the Christie and Loomis figure 60 may be lowered to about 47. It is worth noting that Paul Bert (1878) found that the body temperature of animals fell when kept at low air pressure, *e.g.*, by 2° C for a dog kept for 2 hours at 250, and to 25° C for guinea pigs kept 1 hour at 250 to 220 mm Hg. Such animals in a comatose state and so cooled down may withstand exposure for some time to 3·5% of oxygen = about 27 mm Hg and finally be recovered.

A third series of experiments showed that a mixture of oxygen 93% and carbon dioxide 7% gave results similar to those obtained when pure oxygen

Table I

No. of expt.	No. of animals	Composition of gas	Pressure in chamber, mm Hg	Alveolar pp. of oxygen when dyspnœa occurred	Signs of dyspnœa
			Rats		
1	2	Air	186	28	Deep slow breathing. Convulsive "escape" movements, the limbs straddle out when the animals come to rest.
2	2	99% O	84	37	
3	2	Air	210	33	
4	2	99% O	82	35	
5	2	2% O, 98% N	1792	35	
6	2	Air	210	33	
7	2	99% O	87	40	
8a	2	50% O, 50% N	112	33	
8b	2	50% O, 50% N	122	37	
9	2	99% O	82	35	
10	2	2½% O, 97½% N	1754	45	
11*a*	2	50% O, 50% N	126	39	
11*b*	2	50% O, 50% N	116	34	
12	2	99% O	83	36	
13*a*	2	Air	198	30	
13*b*	2	Air	188	28	
14	2	99% O	91	44	
15	2	99% O	81	34	
16	2	93% O, 7% CO_2	81	32	
17	2	Air	197	30	
18	4	2½% O, 97½% N	1722	42	
19	4	Air	172	25	
20	4	77% O	102	42	
21	4	99% O	72	25	
22	4	93% O, 7% CO_2	72	23	
23	4	99% O	84	37	
			Goats		
1	2	99% O	90	43	The animals while standing showed signs of disturbance and lay down, convulsive movements of limbs occurred below 90 mm Hg.
			Guinea Pigs.		
1	1	Air	190	29	Deep slow breathing. Limbs straddled out and animals fell over.
2	1	99% O	95	48	
3	1	2% O, 98% N	2052	41	
4	2	93% O, 7% CO_2	84	34	
5	2	Air	168	28	
6	2	99% O	91	44	
7	2	93% O, 7% CO_2	96	46	
8	2	99% O	83	36	
9	2	99% O	89	42	
10	2	99% O	84	37	
			Pigeons		
1	1	Air	187	28	Deep slow breathing. Animals sank down and then fell over.
2	1	99% O	78	31	
3	2	2% O, 98% N	1615	32	
4	1	99% O	87	40	
5	1	50% O, 50% N	112	33	
6	1	99% O	84	37	
7	1	2% O, 98% N	1816	36	

Table I—(continued).

No. of expt.	No. of animals	Composition of gas	Pressure in chambers, mm Hg	Alveolar pp. of oxygen when dyspnœa occurred	Signs of dyspnœa
		Monkeys			
1	1	50% O, 50% N ...	105	20	Deep slow breathing. The animals sank down and finally the head fell against the side of the chamber. The animal was unable to balance its position when the chamber was shaken.
2	1	2½% O, 97½% N .	1521	37	
3	1	Air	195	30	
4	1	99% O .	63	17	At this point the monkey was dying.
5	1	99% O ..	65	17	
6	1	99% O ..	80	33	
7	1	7% CO_2, 93% O . .	77	28	
8	1	99% O	79	32	
9	1	3% O, 95% N .	749	35	
10	1	99% O	62	17	Monkey dying.
11	1	99% O	78	31	
12	1	99% O	88	41	

was breathed. Symptoms of dyspnœa came on at 90–80 mm Hg. At such low pressure the alveolar oxygen pressure is not very significantly altered by the 7% of carbon dioxide.

Behague, Garsoaux, and Richet (1928) carried out a series of evacuation experiments which appeared to show that in rabbits exposed to increasing oxygen percentages death takes place at increasing oxygen partial pressures, see Table II.

Table II

% of oxygen in air breathed	Oxygen partial pressure at death
8·15	17
18·21	29
32·42	32
52·61	46·6
61·89	52

If we calculate roughly what the oxygen percentages are, taking 47 for carbon dioxide and water vapour, and use the mean percentage of the figures given in the left-hand column, we get the results shown in Table III.

Table III

Mean % of oxygen	Oxygen partial pressure at death
12	—
20	19·4
37	14·8
56	20·1
75	16·5

The figures in the right-hand column cannot be used to confirm the view taken by these authors, but rather those expressed in this paper.

Margaria (1928) supported the conclusions of Behague, Garseaux, and Richet, and further stated that the addition of carbon dioxide to the atmosphere breathed modified the resistance of guinea pigs to rarified oxygen, but did not affect their resistance to such oxygen-want when this was produced by adding nitrogen to air.

In all the experiments described in this paper the deciding factor is found to be the partial pressure of oxygen in the alveolar air.

Conclusions

When breathing oxygen loss of consciousness occurs in monkeys and men when the barometric pressure falls to about 115 mm Hg. Signs of acute oxygen-want occur in monkeys, goats, guinea pigs, rats, and pigeons when the pressure falls to about 85 mm Hg.

When 5% or less oxygen is breathed at high barometric pressure, symptoms of acute oxygen-want occur when the partial pressure of oxygen in the lungs falls to about 38 mm Hg. The combined partial pressure of water vapour and carbon dioxide in the alveoli of the lungs when these acute signs of oxygen-want occur is the difference between 85 and 38 = 47 mm Hg. This low partial pressure is brought about by deep breathing, fall of body temperature, and diminished production of carbon dioxide.

The author is indebted to Dr. H. J. Taylor for assistance.

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612 . 172

The Rhythm of the Heart Beat. I—*Location, Action Potential, and Electrical Excitability of the Pacemaker*

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(Communicated by Sir Charles Sherrington, F.R.S.—Received August 12, 1933—
Revised April 21, 1934.)

[PLATE 15]

Anatomical and physiological investigations on the heart during the last fifty years have contributed a sound body of knowledge concerning the location of its rhythmic centre (the pacemaker) and the spread thence of excitation. The classical controversy between the exponents of the neurogenic view and of the myogenic view of the origin of the heart beat, may now be regarded as settled in favour of the latter, for the evidence against even the nerve fibre modification of the neurogenic theory is overwhelming, and none of the evidence supporting it stands the test of modern criticism. There remains the more fundamental problem of the nature of those processes in the rhythm centre which are responsible for its recurrent discharge. There has been much speculation concerning them, but no systematic attempt at elucidation.

Engelmann (1895, 1897) postulated a constant weak " inner stimulus," to which the centre is sensitive in varying degree ; this sensitivity (considered to be identical with its sensitivity to electrical stimulation) gradually increases during diastole, until a threshold is reached and discharge occurs. Gaskell (1900, p. 190) expressed an essentially similar view (*cf.* Adrian, 1930), and this view has some support in the evidence of Ishikawa (1924), who found that the excitability of the frog's sinus, but not of ventricle or auricle, increases throughout diastole until the next beat occurs. Langendorff (1902) suggested that the inner stimulus might be provided by the products of dissimilation (Lebensprodukt).

In a series of papers largely theoretical, Hering (1901, 1912, *a*, 1912, *b*) gradually built up a theory of rhythm which is the most elaborate yet attempted. His inner stimulus (Reizbildung) was not continuous, but intermittent with an independent rhythm of its own ; the sensitivity of the centre to this stimulus (Reaktionsfähigkeit), normally identifiable with its electrical excitability, was supposed to follow a time-course initiated by the previous beat. Hering's theory in general lacks experimental foundation, and in particular the independence he assumes for the rhythm of his " Reizbildung " cannot be reconciled with the change of phase produced by premature beats.

Andrus and Carter (1924) found that alterations in the perfusing fluid caused changes in the rate of the perfused heart, and on this inadequate evidence concluded that the rhythmic excitatory process is a disturbance of the ionic equilibrium between the cells and the surrounding fluid. Rothberger (1926) made a similar suggestion, without bringing any further evidence. Finally, von Brücke (1930) saw no justification for postulating a continuous inner stimulus, and attributed the interval between one heart beat and the next partly to refractory period and partly to other inhibitory influences.

In the present series of papers an attempt is made to investigate systematically the behaviour of the pacemaker of the heart, under all those experimental conditions which seem likely to throw light on the nature of the physiological processes underlying its rhythmic production of impulses. In the first paper the pacemaker is located as accurately as possible, and electrical records taken from it. Its recovery of excitability after a normal beat is determined in the usual way by the direct application of induction shocks of varying strengths, and the effect on the recovery curve of vagal slowing and of accelerantes quickening is investigated. The second and third papers deal with the effect of premature beats on the rhythm of the pacemaker, and we hope to publish later an analysis of the action of the vagus.

Method of Experiment

The following account will serve as a general description of the experimental procedures employed throughout the whole investigation. Special modifications will be mentioned in the appropriate sections.

The animal (cat) having been anæsthetized (ether only) was decerebrated by the trephine method and the anæsthetic was then discontinued. A longitudinal skin incision extending from the second to the sixth rib was made two to three centimetres to the right of the sternum. The fourth rib was laid bare and about two centimetres of its sternal end removed after ligation of the intercostal artery. Light artificial respiration by a pump was begun and continued throughout the experiment at just that intensity which allowed the normal respiratory movements to continue. In most experiments the upper lobe of the right lung was ligated at its base and removed in order to give a sufficient exposure of the heart, but this practice was discontinued in the later experiments. The pericardium was opened along a line from the apex of the ventricles to the superior vena cava thus forming a flap which was sewn to the chest wall so as to prevent the lungs from moving over the exposed surface of the heart during respiration. In this procedure the heart rotates somewhat to the left giving a good exposure of the right auricle and the superior vena cava, but care has to be taken to prevent any kinking of the venae cavae.

As electrodes we used small silver hooks soldered on to fine enamelled wires. As far as possible they have been passed through the superficial epicardium only, but doubtless the deeper tissues have also at times been locally injured. When in position the hooks have been closed so that they retained their position despite movements of the heart, and, after packing the aperture in the chest wall with dry cotton-wool in order to keep the wires apart, the edges of the wound have been clipped together. In this way the heart may be investigated with closed chest wall and under conditions as near normal as possible. In the usual arrangement three electrodes have been applied to the heart, one being both the cathode of the stimulating circuit and the earthed lead to the amplifier of the recording circuit. This common lead has been placed as close to the pacemaker as possible (see p. 311), while the respective positions of the anode of the stimulating circuit and of the grid lead have usually been in the region of the tail of the sino-auricular node and on the right auricle, the three leads forming approximately the apices of an equilateral triangle.

Usually both vagi were cut in the neck during the preparation, but in some experiments on vagal tone they were cut during the course of the experiment; rarely they were left intact throughout. The nerves were freed for stimulation by careful dissection so as to preserve the blood supply intact as far as possible, and stimuli were applied through glass-shielded silver electrodes which were sewn in position during the initial preparation.

The nervi accelerantes were approached through the axilla with the fore-limb extended above the head. A skin incision along the inferior border of the pectoralis major was followed by a dissection exposing the second rib, and as much as possible of this was excised subperiosteally. The stellate ganglion was then exposed by blunt dissection, and completely excised except when the nervi accelerantes were to be stimulated, when all the branches of the ganglion except the nervi accelerantes were cut and the latter nerves were freed until their junction with the vagus. The ganglion was left attached merely as a convenience when applying the electrodes, but it was functionally disconnected by pinching the nervi accelerantes at their point of entry. A specially shaped shielded-glass electrode with silver electrodes was then applied to the nervi accelerantes and sewn in position, the skin being then clipped together around the wires leading to the electrodes.

In the first experiments in which both nervi accelerantes were cut, the additional dissection appeared to affect the condition of the animal so unfavourably that we adopted as a usual procedure the excision of the right nerve only. It was later realized that in the decerebrate preparation there is a considerable tonic acceleration through the left nerve alone, so the bilateral operation was again adopted and fortunately was not then found to react unfavourably on the condition of the animal.

After suitable amplification the action potentials from the heart were led to a Matthews oscillograph and photographed by a falling plate camera. In order to shortern the effect of the necessarily large stimulus artefact 0·002 μF coupling condensers have usually been used throughout the amplifier. The resulting great distortion of the action potential was of no consequence as we were only concerned with the action potential as a standard of comparison. In fact the smaller condensers would serve to sharpen the differential features of the records. In most experiments stimulus escape was also reduced by earthing the animal through a metal plate surrounded by cotton-wool soaked in Ringer and placed subcutaneously in the lumbar region. In some experiments the action potentials from an additional pair of leads were led to a string galvanometer and simultaneously photographed on the same plate. In all records time was registered simultaneously by photographing the movements of a small needle attached to the free vibrating end of a 100 d.v. tuning fork.

Premature beats have been set up by break induction shocks from one or two coreless coils, the circuits being broken by a Lucas pendulum which had an electromagnetic release worked by the camera. As a further precaution in minimizing stimulus artefracts in the records a 40,000 ohm non-inductive resistance was placed in the secondary circuit. The stimulus artefact, however, served a useful purpose, as its beginning signalled the exact moment of the stimulus. In the primary were a 12-volt accumulator battery and a variable resistance which was used in adjusting the strength of the induction shock, the coupling between the primary and secondary coils being kept constant. Repetitive stimuli from a neon tube oscillator were applied to the vagus or accelerantes nerves at a rate usually about ten a second.

The temperature of the animal was maintained by the heated table on which it lay. By suitable adjustment this temperature was almost always kept between 36 and 38° C.

A—*The Location of the Pacemaker*

The term " pacemaker " is here applied only to that part of the sino-auricular node in which the impulse originates (*cf.* Lewis, 1925, p. 525; Meek and Eyster, 1914), not to the whole " sinus " as well as the " presinus " (Rijlant, 1932). Lewis, Oppenheimer and Oppenheimer (1910), Sulze (1913), and Lewis, Meakins and White (1914), place it in the head of the sino-auricular node very close to the cavo-auricular angle. Eyster and Meek (1914) come to a similar conclusion in most of their experiments, but in some place it is nearer the middle of the node, in agreement probably with Wybauw (1910). Those results were all obtained with electrical recording, but with one exception no attempt was made to discriminate between points less than 5 mm apart. A more precise localization was claimed by Rijlant (1925, 1931), who in some of his experiments found the initial negativity developing in a circumscribed area about 1 mm in diameter (the " presinus ") between the head of the sino-auricular node and the superior vena cava, while the main activity of the whole node (the " sinus ") followed about 5 σ later.

In the conditions of most experiments the pacemaker appears to remain fixed, but under the influence of strong vagal inhibition it has been shown to move to a position nearer the tail of the node (Meek and Eyster, 1914; Lewis, Meakins and White, 1914).

For the purposes of the present investigation, the electrode common to the stimulating and the recording circuits must be applied as accurately as possible over the pacemaker itself, and the accuracy of its placing must be tested in every experiment. Two methods have been employed :—

Method 1—*Determination of the point associated with the first detectable action potential*

This is the classical method. An extra pair of electrodes is fixed to the right auricular appendage, and connected with a string galvanometer. The action potentials recorded by this instrument are photographed simultaneously on the same plate as those recorded by the oscillograph and provide a fixed point of reference. The earthed lead of the oscillograph amplifier is then applied in various positions on and around the sino-auricular node the grid being on the auricle near the sino-auricular node, and records are taken, each position being noted on an outline drawing of the heart. That position which gives the longest interval between the first deflection of the oscillograph record and the galvanometer record, is nearest to the pacemaker. Thus in fig. 1, Plate 15, observations

2 and 3 are made with the earthed lead in position 4 of fig. 2, and the action potential recorded by the oscillograph begins about 1·5 σ earlier than in observations 1 and 4, corresponding to position 5 of fig. 2. Position 4 is therefore nearer the pacemaker than position 5, and in this experiment it was also found to be nearer than any of the other positions of fig. 2. Subsequent histological examination showed that it lay over the upper part of the head of the sino-auricular node. In two other experiments precisely the same location was found; it corresponds to that of Lewis and his co-workers, and Sulze.

In five experiments, however, the pacemaker was found to lie further down the sino-auricular node, *e.g.*, position 2 of fig. 2. In three of these the string galvanometer was not used, but the main deflection of the oscillograph record was used instead as a point of reference. This appears to be allowable, in experiments such as these in which the grid lead was from a fixed point on the body of the auricle; for the main deflection is not altered appreciably by moving the earthed lead to various parts of the sino-auricular node, and in experiments where the string galvanometer was used the main deflection always bore a constant time relation to the galvanometer deflection. These experiments indicate that the main deflection arises in the auricle and not in the sino-auricular node.

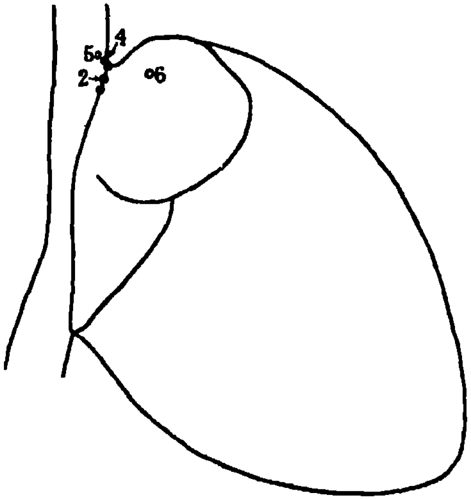

FIG. 2—Drawing of the right auricle and ventricles to show positions of leads. 6 was the grid lead in all observations, and 4 and 5 were the ground leads used for the observations of fig. 1, Plate 15. Position 4 was closest to the pacemaker.

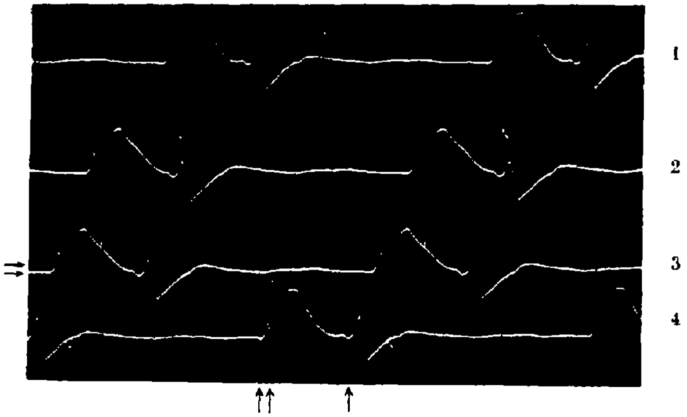

Fig. 1.

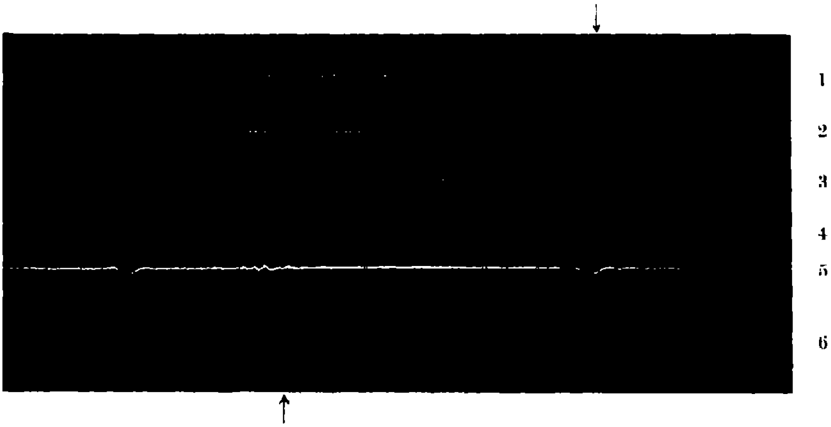

Fig. 3.

Fig. 5.

Fig. 4.

Method 2—*The determination of the point at which a stimulus applied late in the cycle sets up a premature beat which is identical with a normal beat* (*cf.* Lewis, 1910)

The identity can be established by two criteria :

(*a*) The action potential of the premature beat must resemble that of a normal beat. Since the very small coupling condensers of the amplifier (0·002 μF) cause the oscillograph deflections to approximate to the rate of change of electrical potential between the leads, this criterion is a particularly rigorous one.

(*b*) The interval between the first deflection of the normal action potential and its main deflection must be the same as the interval from the stimulus to the corresponding main deflection of the premature beat. This condition is found to hold when a premature beat is set up by a stimulus applied through an electrode (cathode) which Method 1 shows to be close to the pacemaker.

It has been seen above that the main deflection arises from the grid lead on the auricle, so the interval from the first sign of the action potential to the main deflection is the conduction time to the grid lead. The similar time from the stimulus artefact to the main deflection of a premature beat indicates that the stimulus sets up a premature beat after a negligibly short latent period ; a conclusion in agreement with the results of von Frey (1915) and Schellong (1925). When a stimulus falls very soon after the end of the absolutely refractory period, the main deflection of the premature beat may have a latent period even longer than 30 σ, but close inspection with sufficient amplification always reveals small waves throughout this apparently long latency. It would seem that this long latency was due to slow conduction in the relatively refractory period (Drury and Regnier, 1928).

Figs. 3 and 4, Plate 15, exemplify the use of these criteria. In fig. 3 the action potentials of the premature beats in observations 1 and 5 are indistinguishable from the normal action potentials ; in each the interval from stimulus artefact to main deflection is only very slightly shorter than the interval from the first tiny wave of a normal action potential (indicated by an arrow in the more highly amplified observation 6) to its main deflection. It may therefore be concluded that the electrode common to recording and stimulating circuits has lain very close to the pacemaker, since criteria *a* and *b* are satisfied. It was in position 2 of fig. 2. Fig. 4 is similar, but the disturbance of rhythm produced by the premature beats is also shown (see next paper).

In most of the experiments to be described the common lead was first placed in positions 2 of fig. 2, and records made of normal beats, and of premature beats late in the cycle using a stimulus well above threshold. If these records satisfied the criteria of Method 2, the lead was left in that position throughout the experiment; if not, the procedure was repeated with alternative positions until a satisfactory one was found. The more laborious Method 1 was used only in the experiments on the excitability of the pacemaker, where the advantage given by its greater accuracy was highly important. The constancy of the shape of the action potentials obtained throughout a given experiment was taken as sufficient indication that the pacemaker had not materially shifted during the course of the observations.

B—*The Action Potential of the Pacemaker*

Some physiological process in the pacemaker must be conceived of as preceding and conditioning the production of the impulse, and it is possible that this process might be associated with a slight and gradual change of electrical potential preceding the rapid potential changes produced by the propagation of the impulse. With this in view, records have been made with large coupling condensers of 4 μF in the amplifier; the deflection produced by a constant potential then fell to half its initial value in slightly less than one second. Fig. 5, Plate 15, is a record obtained in this way, with the leads to the amplifier in positions 4 and 6 of fig. 2. It shows two auricular action potentials with the ventricular action potential following the first. There is no slow change of electrical potential preceding the sudden upward deflection (shown by the arrow) which signals the propagation of the impulse. The small slow wave between the ventricular and the next auricular action potential is the ventricular T wave. Similar results have been obtained in four other experiments.

These results are in agreement with Adrian's (1931) observations on the frog's sinus. However, still more refined technique may yet detect some changes [*cf.* Adrian and Gelfan's (1933) results with rhythmic beats of skeletal muscle fibres], but artefacts due to the incessant movement of the tissues will be difficult to exlcude. At present it is clear that any electrical changes possibly present in the latter part of diastole can only be of a very much smaller order than those associated with the appearance and propagation of the impulse.

C—*The Electrical Excitability of the Pacemaker during its Rhythmic Cycle*

No previous attempt appears to have been made to measure the electrical excitability of the mammalian pacemaker itself. Observations on other parts of the heart have shown that a beat is followed in turn by an absolutely refractory period and then a relatively refractory period, but opinion is divided as to whether in the last part of diastole the excitability continues to increase or attains a constant level. In frog's auricles and ventricles, Trendelenburg (1911), Adrian (1921), Ishikawa (1924), Schellong and Schütz (1928) and Buchthal (1931) found a longer or shorter period of constant excitability at the end of diastole, while Engelmann (1895) found none. Ishikawa (1924) found that the excitability of the frog's sinus increased till the end of diastole. In mammalian auricle and ventricle, Cushny and Matthews (1897) found a rising excitability at the end of diastole, but Cushny later (1912) failed to confirm that result. Drury and Regnier (1928), using mammalian auricles driven by direct stimulation, found a fairly long period of constant conductivity in the latter part of diastole.

We have used the technique already described in an attempt to measure the excitability of the pacemaker itself. In every experiment the electrode common to stimulating and recording circuits was placed over the pacemaker, using Method 1 (p. 311). Single break induction shocks of varying intensity were applied at random in all phases of the cardiac cycle, and with each strength of stimulation records were taken until the critical point at which recovery was just sufficient for the stimulus to excite was fairly accurately fixed. From these results the usual recovery curves were constructed.

(*a*) *The Normal Recovery Curve*—Fig. 6 is a typical example of the curves obtained in 12 out of 17 experiments. The strength of the stimulus is plotted along the ordinate, and its time of application along the abscissa; the latter is measured by the interval between the beginning of the action potential of the previous beat and the stimulus artefact. For each strength of stimulus two points are plotted, the earliest time at which it evokes a premature response, and the latest time at which it fails to do so. These are joined by a short horizontal line, and the whole recovery curve is a smooth curve passing as nearly as possible through these lines. Its lower end is seen to run horizontally through the latter part of diastole at a strength of stimulus just on the threshold, *i.e.*, it sometimes excited and sometimes failed to excite a response. Fig. 7 is a similar example.

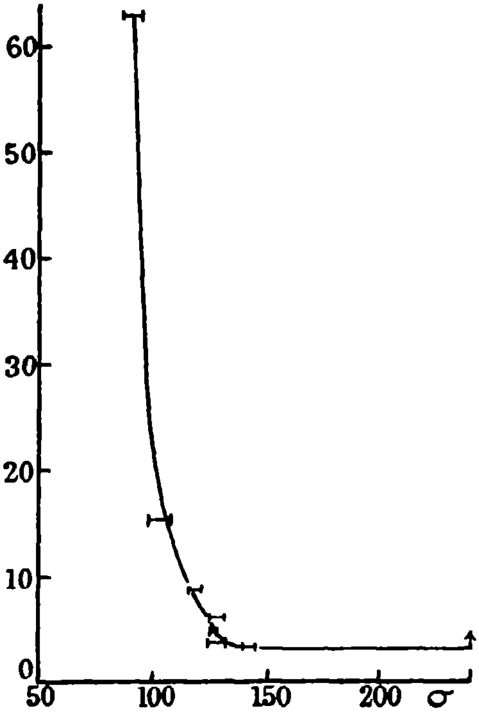

Fig. 6—Recovery curve of "pacemaker." Ordinates, strength of stimuli in arbitrary units, abscissæ intervals in sigma after the beginning of the previous normal beat. The arrow shows the time of the next normal beat. Each horizontal line joins the longest interval at which that stimulus failed to excite to the shortest interval at which a premature beat was set up.

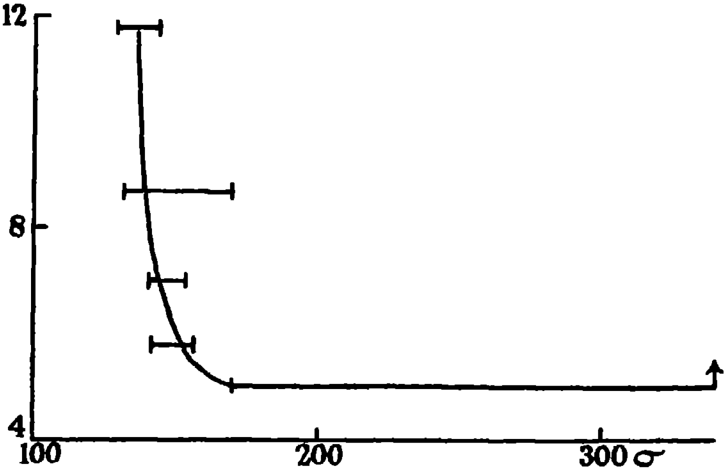

Fig. 7—Recovery curve of "pacemaker" in the same experiment as figs. 1 and 2. The cathode of the stimulating circuit was in position 4 of fig. 2.

In the remaining 5 of the 17 experiments, of which fig. 8 is an example, the threshold increased during the latter part of the cycle, *i.e.*, the excitability reached its maximum relatively early in diastole and then decreased progressively until the end of the cycle. A similar "supernormal" phase of excitability was described by Adrian (1920) and Wastl (1922) in frogs' ventricles. It was not affected by tetanic stimulation of the accelerantes, and could not be attributed to faulty artificial ventilation, for it occurred when this was so adjusted that normal respiratory movements were present.

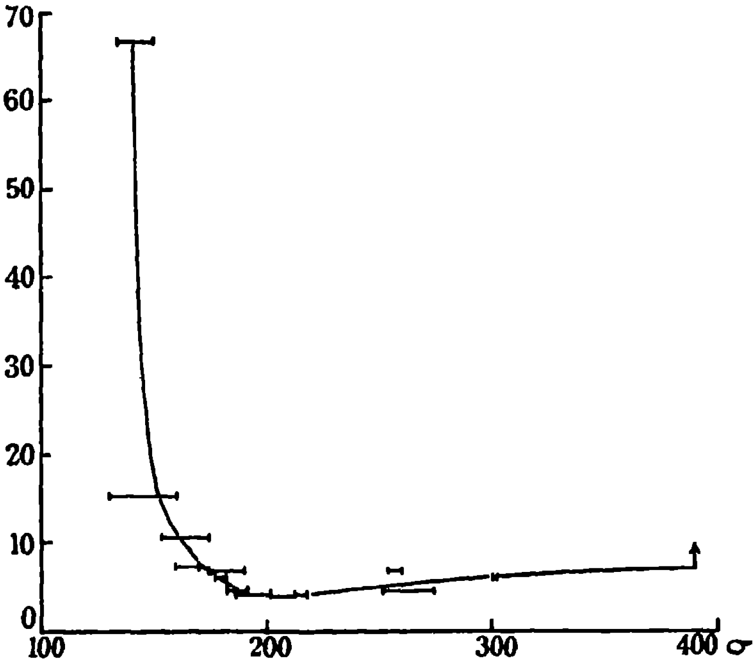

FIG. 8—Recovery curve of "pacemaker" in another experiment.

A possible source of error in observations such as these performed on a moving tissue must be mentioned, namely, variations in tissue resistance and in shunting through inexcitable parts caused by the movements. Three rough determinations of the resistance of the tissues between the stimulating electrodes showed that its average value was 3500 ohms, with a variation of about 500 ohms during the cycle. In the presence of a 40,000 ohm resistance in the secondary circuit, the effect of this variation on the strength of the stimulus would be negligible. Variations in the amount of shunting by such inexcitable structures as connective tissue or blood in the auricular cavity, cannot be measured, but the constancy of the threshold in the latter part of diastole in the 12 experiments is presumptive evidence that these variations are not a serious source of error.

The 17 experiments therefore provide clear evidence that, in the later part of diastole, the electrical excitability of the structure stimulated remains constant, or passes through a supernormal phase and then slightly decreases : in any event it does not rise as the next spontaneous beat approaches. It remains to ask, what exactly is the structure stimulated ? The common electrode was placed with all care as near as possible to the pacemaker, and it may reasonably be assumed that in some experiments at least the pacemaker was directly stimulated. But the stimulating current would also have passed through adjacent fibres of the sino-auricular node, and, if at any time these fibres possessed a lower threshold to electrical stimulation than the pacemaker itself, then the observations made at that time concern these fibres and not the pacemaker. We have no means of detecting whether or when this happens, but the behaviour of such rhythmic centres is probably very similar to the actual pacemaker, which is generally believed to be that particular rhythmic centre possessing the most rapid intrinsic rhythm. Thus it may reasonably be assumed that any changes found in the excitability of these more highly excitable fibres will be similar to changes occurring in the adjacent pacemaker itself. We may therefore treat the curves as recovery curves of the pacemaker.

(*b*) *Influence of Tetanic Accelerantes Stimulation on the Recovery Curve*—This was investigated in seven experiments. In all the acceleration of rhythm was accompanied by a shortening of both the absolutely and relatively refractive periods ; the total shortening, expressed as a fraction of the total refractory period, was less than the shortening of the cycle, expressed as a fraction of the whole cycle. In the example shown in fig. 9 the cycle was reduced by 16%, the refractory period by about 6%. In some experiments, though not in that of fig. 9, the threshold reached at the end of the relatively refractory period was lowered a little by the accelerantes stimulation.

In a complementary experiment, the spontaneous accelerantes tone was abolished by division of the nerves. The cycle lengthened from 259 σ to 348 σ (34%) and the refractory period from 127 σ to about 150 σ (18%).

The results are in line with those of Andrus and Carter (1930), who found that adrenalin shortens the absolutely refractory period of cardiac muscle by as much as 40%.

(*c*) *Influence of Tetanic Vagus Stimulation on the Recovery Curve*—This was investigated in 14 experiments, in all of which submaximal stimulation was used, insufficient in intensity to cause a shift of the pacemaker to some other part of the sino-auricular node—a change easily recognized by the abrupt alteration in the shape of the action potential (p. 311). The lengthening of the cycle varied from 15% to over 50%. The effect on the recovery curve was variable.

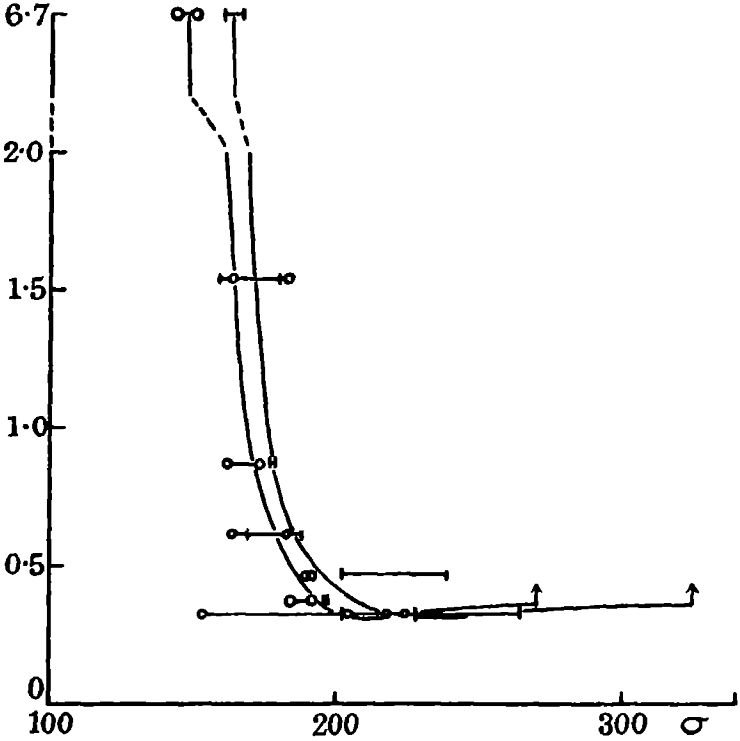

FIG. 9—Recovery curve of "pacemaker," but in addition recovery curve obtained during a tetanic stimulation of accelerantes, which shortened the cycle from 325 σ to 270 σ as shown by the two arrows. The circles represent the intervals obtained during the accelerantes stimulation.

In six experiments, one of which appears as fig. 10, the curve remained substantially unaltered, and in two others there was a doubtful slight shortening of the refractory period.

In the other six experiments the refractory period was shortened by amounts varying from 6%, fig. 11, to 50%. When the shortening was pronounced,

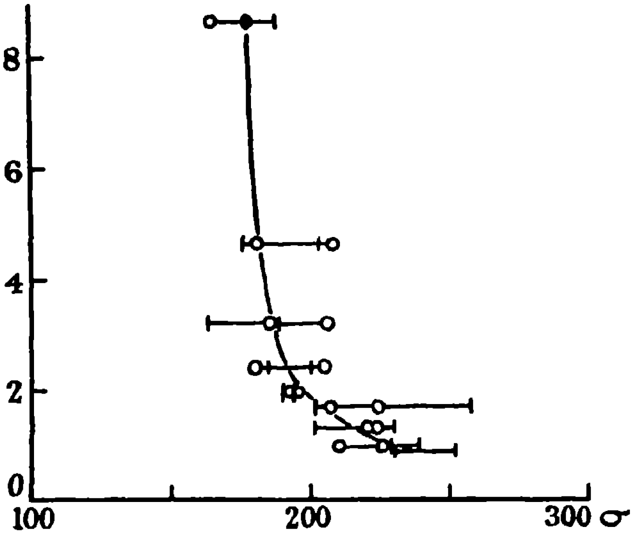

FIG. 10—Recovery curves of "pacemaker" both normal and during a vagal slowing of the heart which lengthened the cycle from 350 σ to 530 σ. The circles represent the intervals obtained during the vagal slowing. The recovery curve seems unaltered.

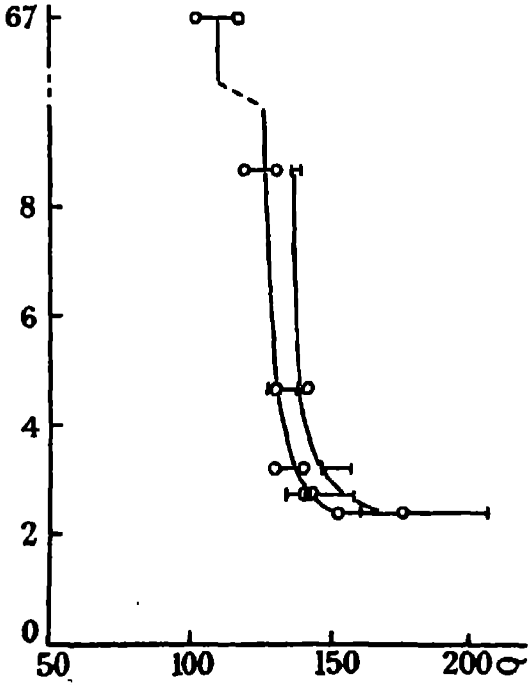

FIG. 11—Recovery curves of "pacemaker" as in fig. 10 but in another experiment, the vagal slowing lengthening the cycle from 290 σ to 450 σ and being accompanied by a slight shortening of refractory period.

the threshold reached and maintained during the period of full recovery was definitely lowered.

Taking all the experiments together, we found no relationship between the degree of vagal slowing and the change in refractory period. Twice, indeed, it was clear that a small change in rate during an experiment can be accompanied by a large change in refractory period: thus, after vagus stimulation had lengthened the cycle from 360 σ to 630 σ (+ 75%) and shortened the refractory period from 190 σ to 114 σ (− 40%), the length of the cycle spontaneously diminished to 550 σ (+ 53%) through vagal fatigue, and at the same time the refractory period increased to 182 σ (− 4%), almost its original value.

The variability in the effects obtained with vagus stimulation might be due to small differences in the placing of the stimulating electrodes causing the sino-auricular node to be stimulated directly in some experiments and only the auricular muscle in others. This would disturb the results only if the vagus stimulation has a grossly different effect on the auricular muscle. We tested this effect deliberately in three experiments: in two of them the rate was slowed by 30% to 40% and the refractory period of the auricular muscle remained unchanged; in the third, the rate was slowed by 43% and the refractory period of the muscle shortened from 180 σ to 75 σ. In the latter, the refractory period of the pacemaker was shortened almost to the same extent, for the premature beats, with the possible exception of the one at 75 σ, disturbed the rhythm of the pacemaker (see p. 356). It seems, then, that the pacemaker and the auricular muscle are similarly affected by vagus stimulation, and that the variability of the present results is not due to inaccurate placing of the electrode.

Lewis, Drury and Bulger (1921) and Andrus and Carter (1930) found that tetanic vagus stimulation invariably produced a great shortening of refractory period in the mammalian auricle. The constancy of their results may be due to the use of stronger vagus stimulation than we employed. On the other hand, Ishikawa (1924) found that vagus stimulation had no effect on the recovery curves of frog's sinus, auricle, and ventricle.

(*d*) *The Recovery of Excitability after Premature Beats*—This was examined in 14 experiments, by applying to the pacemaker two stimuli, separated by a varied short interval. The first stimulus set up a premature beat, and the second tested the recovery of excitability after this beat. The refractory period following the premature beat was always shorter than normal, and its shortening was greater when the curtailment of the previous cycle was greater. Fig. 12 shows approximately a normal recovery curve (*cf.* fig. 6) and a recovery curve after a premature beat which curtails the previous cycle by one half. The curves are almost parallel; between them would lie the corresponding curves obtained after less curtailment of the previous cycle. Fig. 13 also shows recovery curves after a normal and an early premature beat; a supernormal phase is present, and moves earlier with the shortening of the refractory period.

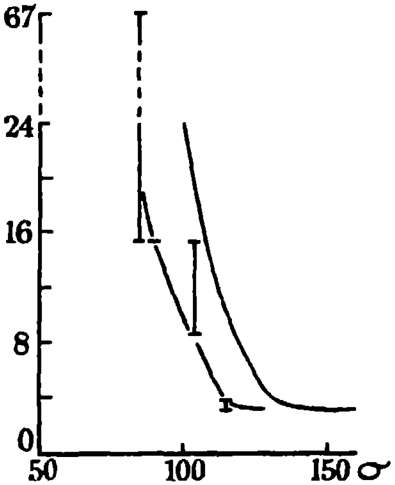

FIG. 12—Recovery curves of "pacemaker," the one on the right side being the normal recovery curve (see fig. 6 for details of this), and the other the recovery curve after a premature beat set up after a curtailed cycle equal to 0·5 of a normal cycle. This curve is approximately drawn through the perpendicular lines which for each interval join the weakest stimulus which excited to the strongest stimulus which failed to excite. At an interval of 90 σ this perpendicular line had no length, for at a strength of 15·4 the stimulus sometimes excited and sometimes failed to excite.

The earlier recovery of excitability after a premature beat was discovered by Trendelenburg (1903) in frog's ventricle, and observed by de Boer (1916), Ishikawa (1924), Umrath (1925), Lewis and Master (1925), Schellong and Schütz (1928) and Buchthal (1931), the last two investigations showing that the shortening of refractory period corresponded with the shortening of the action potential of the premature beat.

Discussion

Any plausible theory of the production of rhythmic impulses by the pacemaker, must postulate some sort of continuous process or change in the pacemaker during the interval between two impulses. From the observations that have been described two general statements, both of a negative character, may be made about the nature of that process.

The first statement is that *no demonstrable relationship exists between the course of that process, and the electrical excitability of the pacemaker.* This rests on the following observations :—

1. The electrical excitability in the last third (approximately) of the normal cycle either does not alter, or shows a slight decline subsequent to a supernormal phase (Section C, (*a*)). All this time the hypothetical process must be continuing.

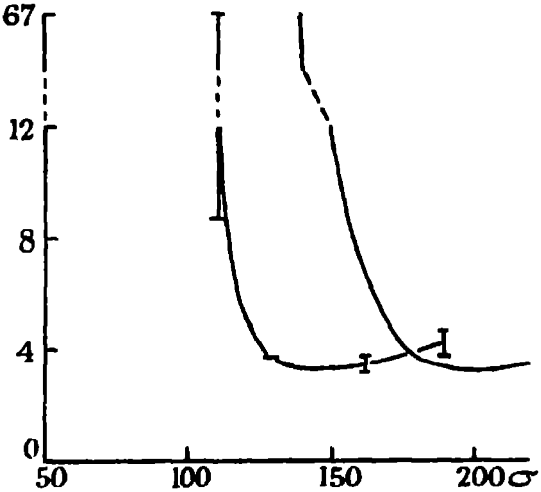

FIG. 13—As in fig. 12 in another experiment.

2. When the time-course of the hypothetical process is shortened by excitation of the accelerantes, the refractory period to electrical excitation of the pacemaker is also shortened; but, when the time-course of the hypothetical process is lengthened by vagus excitation, no lengthening of the refractory period is found. On the contrary, it is often shortened, and that to a highly variable degree (Section C, (*b*), (*c*)).

3. In the cycle initiated by a premature beat, *i.e.*, the premature beat cycle, the time-course of the hypothetical process is lengthened (see p. 335), but the refractory period is shortened (Section C, (*d*)).

The absence of relationship between the rhythmic process and extrinsic electrical excitability was inferred by Hering (1907) from the fact that some drugs which stop the heart-beat increase the excitability. Cushny drew the same conclusion from evidence resembling that of observation 1 above. The present observations, however, with the application of stimuli as near as possible to the seat of the rhythmic process (the pacemaker) itself, place the generalization on a more secure basis. The only previous attempt to investigate directly the excitability of a rhythmic centre was Ishikawa's (1924) work on the frog's sinus, where he found that excitability increased through diastole right up to the appearance of the next beat; in this respect the frog's sinus and the cat's sino-auricular node would seem to differ.

The second statement is that, *in the interval between two impulses, the hypothetical process in the pacemaker does not betray itself by giving rise to any detectable electrical potential* (Section B).

According to the membrane theory an impulse in nerve or muscle is propagated as a change of polarization and permeability in certain membranes, and an impulse is set up by an extrinsic stimulus, *e.g.*, an induction shock, when that stimulus produces a sufficiently great diminution in the polarization of the membrane in question (the conducting membrane). An attractive explanation of the intrinsic setting up of an impulse, *e.g.*, the origin of a normal beat in the pacemaker, would be that the polarization of the conducting membrane suffered a progressive diminution until it passed the critical point and an impulse was then set up. But then one would expect the setting up of this impulse to be preceded by :—

(*a*) a slowly developing electrical potential, the pacemaker becoming increasingly negative, and

(*b*) a gradual change in electrical excitability, the pacemaker becoming more and more easily excited; and these changes should continue right up to the moment at which the beat was set up.

Since neither of these effects are observed experimentally, the setting up of the impulse cannot be preceded by a gradual diminution in the polarization of the conducting membrane. The changes in this membrane signalled by the beginning of the action potential of the beat must be impressed on it by some other mechanism in which the gradual preliminary changes must have been occurring. It is this mechanism which is the seat of the rhythmic activity of the pacemaker. In the following papers this *rhythmic mechanism* will be subjected to a more direct investigation.

Summary

This paper is the first of a series in which an attempt has been made to investigate the mechanism responsible for the rhythmic production of beats by the pacemaker of the heart.

The position of the pacemaker has been determined by the usual methods. In some experiments it has been found to be situated in the head of the sino-auricular node (agreeing with Lewis and Sulze), but in other experiments it has been located more towards the middle of the node.

No potential changes have been detected in the pacemaker preceding those produced by the impulse, hence it is concluded that the processes responsible for setting up the beat are not associated with potentials of the same order of magnitude as those produced by the propagation of the impulse.

The recovery of excitability at various stages after a normal beat has been tested by applying at random stimuli of varying strengths. The recovery curves constructed from such experiments show the following features.

1. The absolutely refractory period usually lasts for about half the cardiac cycle and the relatively refractory period is very short, so that maximum excitability is always reached before two-thirds of the cycle have elapsed.

2. During the last third of the cycle the excitability usually remains constant, but it may decline so that a supernormal phase is present.

3. Stimulation of the nervi accelerantes slightly quickens all phases of the recovery curve.

4. Stimulation of the vagus may also quicken the recovery, but quite marked vagal slowing may be accompanied by no change in the recovery curve.

5. The recovery of excitability is always quickened after a premature beat, though the cycle after that beat is longer than normal.

On account of this complete lack of correlation between the recovery of excitability of the pacemaker and its spontaneous setting up of beats, it is concluded that the stimuli do not act directly on that part of the pacemaker responsible for this rhythmic production of beats, *i.e.*, on the rhythmic mechanism of the pacemaker. Since the setting up of a beat is not preceded by any detectable action potential, it is concluded that the rhythmic mechanism is not situated in the surface membranes of the muscle fibres composing the pacemaker.

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EXPLANATION OF PLATE

Fig. 1—A series of records of action potentials from the beating heart. The white lines are records from the Matthews oscillograph, and the black lines (indicated by upper arrow, observation 3) show simultaneous string galvanometer records with both leads in a constant position on the body of the auricle. The grid lead to the Matthews oscillograph was in position 6 of fig. 2, while the earthed lead was in position 5 for observations 1 and 4 and position 4 for observations 2 and 3. The first arrow below the fourth observation indicates the small initial action potential arising from the sino-auricular node, the second arrow indicating the large auricular action potential, and the third arrow the ventricular action potential. The coupling condensers have a capacity of 0·02μF. This and all subsequent records read left to right, and an

upward deflection indicates negativity of the earthed lead, *i.e.*, the lead from the pacemaker. Tuning fork, 1 d.v. = 10σ.

FIG. 3—*A series of action potentials of normal and premature beats recorded by the Matthews oscillograph.* In observations 1 and 5 premature beats were set up by an induction shock, the cathode of the stimulating circuit being also the earthed lead of the recording system. The main auricular deflection of the action potential occurs after the record of the stimulus artefact (indicated by the arrow above observation 1). In observation 2 there is a record of the stimulus artefact which was too soon after the previous beat to set up a premature beat. The irregular series of deflections following this artefact is the ventricular action potential corresponding to the previous normal beat. Observation 6 is a more highly amplified record of a normal beat. Coupling condensers = 0·002μF.

FIG. 4—A series of observations similar to fig. 3 but taken with a slower plate in order to show the disturbance of rhythm produced by premature beats (indicated by arrow).

FIG. 5—Normal action potentials with leads in positions 4 and 6 of fig. 2. Coupling condensers = 4μF. The first arrow indicates the abrupt onset of the action potential and the second arrow indicates the ventricular T wave.

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The Rhythm of the Heart Beat. II—*Disturbance of rhythm produced by Late Premature Beats*

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The disturbance of cardiac rhythm by a premature beat was first studied by Marey (1875), who found that the lengthening of the cycle following the premature beat usually just compensated for the shortening of the cycle preceding it, the two cycles together being equal to two normal cycles. The phase of the rhythm therefore suffered only a transient disturbance by the premature beat. Engelmann (1895) obtained similar results when premature beats were set up by stimulation of the frog's ventricles, but later (1897) he stated that there was no lengthening of the cycle following the premature beat set up by stimulation of the vena cava. On the basis of these observations he put forward the suggestion that the ventricle beats in response to discrete impulses discharged rhythmically from the sinus. A premature beat set up by stimulation of the ventricle would be prevented from reaching the sinus by the next normal impulse from there, and so the essential rhythm of the heart (the sinus rhythm) would remain undisturbed, *i.e.*, there would be a completely compensatory pause following the ventricular premature beat. Cushny and Matthews (1897) working for the first time with premature beats of mammalian hearts found that an auricular premature beat was only followed by a compensatory pause when set up relatively late in a cardiac cycle. The pause following earlier premature beats was too short to be compensatory. Hering (1900) confirmed both Engelmann and Marey by finding complete compensation after ventricular premature beats, but this was rare with auricular premature beats and he stated that premature beats set up by stimulation of the vena cava were followed by a pause equal to a normal cycle (*cf.* Engelmann), though his figures show a slight lengthening.

Wenckebach (1903, 1904) elaborated the suggestion of Engelmann (1897) in order to explain the condition of partial compensation which is especially frequent with auricular premature beats set up early in diastole. Such premature beats would be conducted to the pacemaker and would excite it before the next normal beat was due to be set up. This conducted premature beat of the pacemaker would be similar in effect to a beat directly set up, *i.e.*, it would be followed by a cycle of normal length, the phase of the rhythm would be altered, and the pause following the auricular premature beat would not be completely compensatory. However, this explanation would not account for the finding of Cushny and Matthews that such incompletely compensatory pauses are the longer the earlier the premature beat. Wenckebach therefore made the additional assumption that the conduction rate in the auricle was slowest early in diastole and gradually became more rapid. This has since been experimentally proved by Lewis and Master (1925) and by Drury and Regnier (1928).

Despite certain criticisms notably by Hofmann and Holzinger (1912), but also by Cushny (1912), Sansun (1912), Lewis and White (1914), Miki and Rothberger (1922), and Lewis (1925), Wenckebach's explanation still is in satisfactory agreement with almost all the reliable experimental observations that have been obtained (*cf.* Rothberger, 1926; Lewis, 1925). Neither Hofmann and Holzinger's nor Cushny's results were obtained with normally beating intact hearts. The one experiment of Miki and Rothberger which seemed to offer the most difficulty to Wenckebach's explanation was undoubtedly complicated by stimulation of the vagal inhibitory fibres (see p. 334). Sansun as well as Lewis and White attempted to stimulate the pacemaker and found that the cycle following a premature beat was longer than normal, but Lewis and White thought that this deviation from Wenckebach's explanation could be due to inaccurate placing of the electrodes on the pacemaker, and little weight need be attached to Sansun's deviations which were barely significant with his method of recording. Moreover, Lewis, Meakins, and White (1914) actually produced an experimental confirmation of Wenckebach's explanation by directly measuring conduction times.

Hofmann and Holzinger (1912) give the clearest exposition of an alternative hypothesis. They believe that a premature beat of the pacemaker is followed by a pause longer than normal, and that the earlier the premature beat the longer is this pause, for the condition of "exhaustion" following such a premature beat is the greater the earlier it is. Miki and Rothberger (1922) give an unconvincing discussion of their own results and come to the conclusion that Wenckebach's explanation holds for premature beats conducted to the pacemaker, but with premature beats directly set up they state that there is also an "inhibition," which they fail to distinguish from the inhibition produced by stimulation of vagal inhibitory fibres (p. 334). Rothberger later (1926) adopts Wenckebach's explanation entirely, but Lewis (1925) thinks that Miki and Rothberger's experiments offer a difficulty to the complete acceptance of Wenckebach's explanation.

An attempt has here been made to clear up the present unsatisfactory state of the subject by systematic study of the effects produced by direct stimulation of the pacemaker in a heart beating spontaneously under conditions as near normal as possible. The method adopted was that described in the previous paper, p. 308.

I—EXPERIMENTAL INVESTIGATION

A—*The Rhythm of the Heart Beat*

Mines (1913) called attention to the great regularity of the rhythm of the frog's heart beat, and by means of an ingenious apparatus was able to detect very small changes in the rhythm. He found that the variations in the lengths of the successive cycles did not exceed 1%. We have found that the denervated cat's heart is still more regular. Usually successive cycles do not vary by more than 0·5% and frequently the variation is less than 0·2%, which is about the limit of measurement in our records. As a consequence the investigation of heart rhythm may be conducted with a relatively high degree of accuracy.

B—*Disturbance of Rhythm produced by a Single Premature Beat*

1—*Introduction*—Fig. 4, Plate 15, of the previous paper and fig, 1 show observations in which single premature beats have been set up at various phases of the normal rhythmic cycle of the heart by a stimulus applied as near as possible to the pacemaker. It will be seen that the premature beat has produced a disturbance in the rhythm of the pacemaker, the length of the cycle after the premature beat being obviously longer than a normal cycle. Accurate measurements (Tables I and II) also show that even the next cycle may be slightly lengthened, but thereafter the normal rhythm is resumed and continued indefinitely. In discussing this disturbance of rhythm a definite terminology is needed, and the one here adopted is shown in fig. 1 for "*single premature*" *beats*, and in fig. 2 for a "*second premature*" *beat* curtailing the first premature beat cycle (see the second part of this paper).

In the next paper it will be seen that as a premature beat is introduced progressively earlier in a normal cycle, *i.e.*, as the curtailed normal cycle is progressively shortened, there is with a very short curtailed cycle an abrupt transition to an absolutely different disturbance of rhythm. Premature beats are classified as *early-premature-beats* and *late-premature-beats* according as they are earlier or later than this transition point. This paper is concerned exclusively with late-premature-beats, and they will for shortness be called simply premature beats.

2—*General Description of Relationship between Durations of Curtailed Cycles and Late Premature Beat Cycles*—In Table I are given the measurements of a series of observations each value being expressed as a fraction of the preceding normal cycle. As the curtailed cycle shortens the premature beat cycle progressively lengthens, and there is also a slight lengthening of the subsequent cycle. Although the normal rhythm is resumed after the subsequent cycle, its phase is altered, for the lengthening of the premature beat cycle and of the subsequent cycle (where this occurs) does not completely "compensate" for the shortening of the curtailed cycle.

In many experiments there is no appreciable lengthening of the subsequent cycle, and in no experiment does it amount to more than one-fifth of the lengthening of the premature beat cycle (*cf.* Table II). Its consideration will therefore be deferred (p. 340) until the lengthening of the premature beat cycle has been fully dealt with.

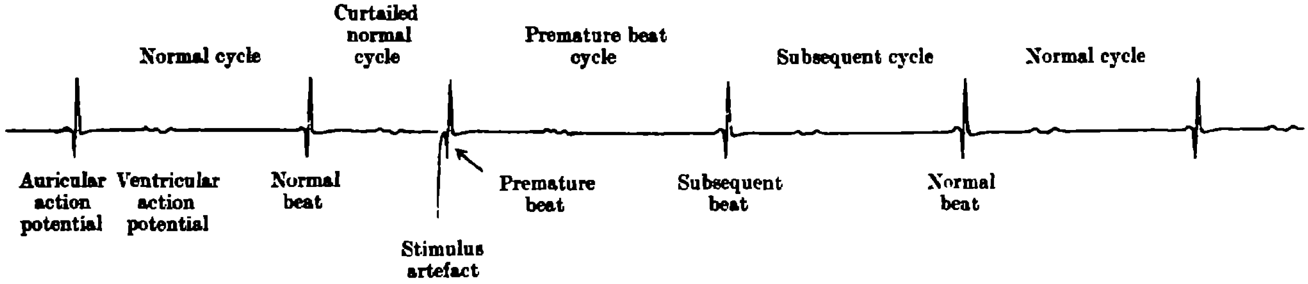

FIG. 1—Tracing of a photographic record of action potentials (one lead on pacemaker and other on auricle), showing disturbance of rhythm produced by a single premature beat (*cf.* fig. 4, Plate 15).

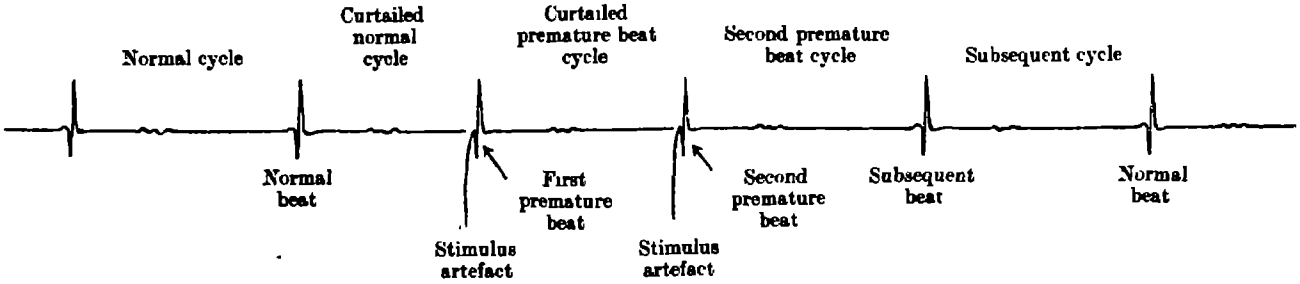

FIG. 2—As in fig. 1, but with a second premature beat curtailing the first premature beat cycle.

Table I—Single Premature Beats

Curtailed cycle	Premature beat cycle	Subsequent cycle
0·935	1·035	1·00
0·925	1·05	1·00
0·81	1·085	1·00
0·795	1·105	1·005
0·745	1·105	1·00
0·71	1·12	1·01
0·67	1·125	1·01
0·67	1·125	1·01
0·66	1·125	1·01
0·615	1·13	1·005
0·61	1·13	1·005
0·595	1·14	1·01
0·58	1·145	1·01
0·56	1·145	1·005
0·545	1·155	1·01
0·525	1·16	1·025
0·46	1·165	1·005

Graphical representation has been adopted in order to study in detail the relationship between the durations of the curtailed cycles and of the corresponding premature beat cycles. In fig. 3 the results of Table I have been plotted, the durations of the curtailed cycles being ordinates and time abscissæ.

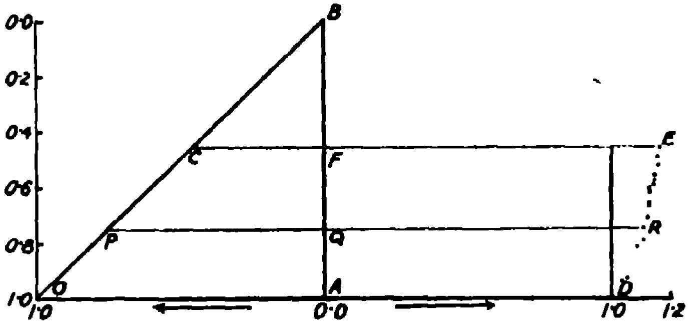

Fig. 3—Plotting of results to show relationship between durations of curtailed and premature beat cycles. Curtailed cycles plotted as ordinates from B to A and premature beat cycles as abscissæ from A to D. All values are expressed as fractions of the normal cycle. Further details in text.

The premature beats have been synchronized so as to fall on the line AB perpendicular to the X axis. The preceding normal beats will therefore lie on the line OB where OA = AB = one normal cycle, and the subsequent beats are seen to lie along the line DE. The temporal course of events in each observation is given by a line drawn parallel to the X axis. Thus with the line PQR, P is a normal beat, Q the premature beat, and R the subsequent beat, PQ being the curtailed cycle and QR the corresponding premature beat cycle.

In Table I and fig. 3 it will be seen that progressively longer curtailed cycles are followed by shorter and shorter premature beat cycles, until with a curtailed cycle just shorter than a normal cycle the premature beat cycle is just longer than a normal cycle. In the limiting case, therefore, the rhythm is not disturbed by a beat set up simultaneously with a normal beat, *i.e.*, OA = AD = one normal cycle. Similar relations between curtailed and premature beat cycles have been obtained in all of the 35 experiments in which single premature beats have been set up. In fig. 4 the line DE shows the curve for the experiment with the greatest lengthening of the premature beat cycle (1·25 times normal), and DF for that with the least (1·05 times normal). In all experiments the curve has been smooth with a convexity to the right.

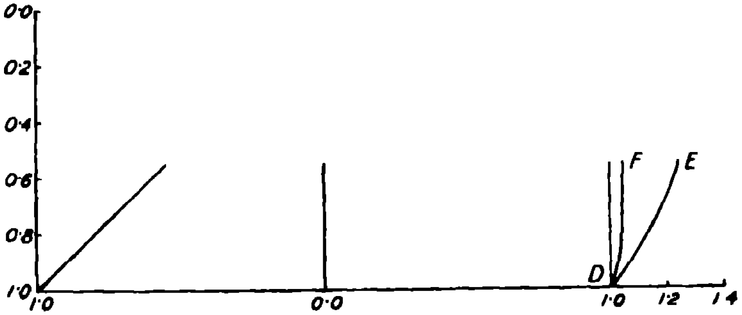

Fig. 4—Plotting as in fig. 3. The line DF shows the curve for the least lengthening of the premature beat cycle, and DE the curve with the greatest lengthening.

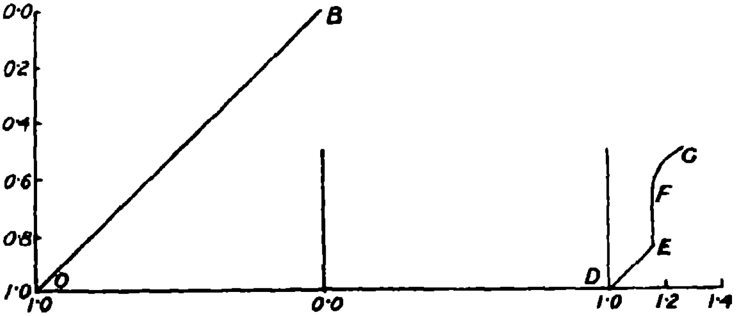

Fig. 5—Plotting as in fig. 3. The curve DEFG shows the curve for the premature beat cycles as predicted according to Wenckebach's hypothesis. See text.

3—*Wenckebach's Explanation*—Can this lengthening be reconciled with Wenckebach's explanation by assuming an inaccurate placing of the stimulating electrodes on the pacemaker as suggested by Lewis and White in discussing the significance of their own somewhat similar results ? According to Wenckebach's explanation the curve from D (fig. 5) should be parallel to OB for a distance DE equal to the conduction time to the pacemaker, and should then follow a course dependent on the changes in the conduction time in the various stages of recovery existing for each length of curtailed cycle. Drury and Regnier (1928) found that the conduction rate of the mammalian auricle recovered to normal in about 0·17 seconds after a previous beat, and so lengthening of conduction time could only exist for observations with very short curtailed cycles. This has been confirmed in the present series of experiments for the conduction rate has been observed to be constant during the last 0·25 of the cycle. The curve would, therefore, have a section EF perpendicular to the X axis until the curtailed cycles became so short that the conduction time lengthened giving rise to the section FG. Shift of pacemaker after the premature beat (Lewis, 1925, p. 225) would produce further modifications in the curve, but it only occurred in a few observations. The dissimilarity between fig. 5 and the curves obtained in all our experiments is so obvious that no detailed consideration is necessary. In none was there the sharp angle DEF or the concavity EFG to the right. In several experiments there was a portion DE parallel to OB—in five for more than 0·05 of the normal cycle (*cf.* Table II), but in none did it terminate in an angle DEF, and in no experiment was there the concavity EFG to the right. Wenckebach's explanation, therefore, will not do.

The above parallelism of the experimental curve to OB for longer than 0·05 of a cycle from its origin may be due to the premature beat being set up by a stimulus applied some distance from the pacemaker, but this explanation seems inadequate as the distance would have to be as long as 1 cm to account for such results. Usually the phase of the rhythm changes after a premature beat set up with a curtailed cycle as long as 0·98. This shows that the premature beat is set up close to the pacemaker, but the location of the pacemaker by this method (*cf.* Hirschfelder and Eyster, 1907) is not very precise, because in any case the initial part of the curve, *e.g.*, DE in figs. 3 and 4, deviates very slightly from parallelism with OB.

4—*Stimulation of Terminal Fibres of Vagus*—Is the lengthening of the premature beat cycle due to inhibition resulting from direct stimulation of the vagal nerve fibres by the stimulus setting up the premature beat ? The effects of stimulation of these nerve fibres have been described by Gaskell (1883), Engelmann (1895), and Miki and Rothberger (1922).

In five of our experiments it was noticed that stimuli falling in the absolutely refractory period, *i.e.*, stimuli which failed to set up a premature beat, caused a lengthening of several normal cycles, which was most marked in the first cycle. When a stimulus fell later in a cycle and set up a premature beat, there was a similar lengthening of the cycles following the premature beat cycle. Now the time course of this lengthening corresponds closely with that produced by stimulating the cervical vagus with a single induction shock (Brown and Eccles, 1934), the latent period of the effect being, however, about 20 σ longer in the latter case. A particularly favourable example for this comparison was one in which a considerable slowing of the heart was produced by a stimulus which was too weak to give rise to a premature beat. It may therefore be concluded that the slowing observed in these five experiments is due to direct stimulation of vagal nerve fibres in the heart muscle. Vagal nerve fibres could not have been excited in those experiments where the rhythm was unaffected by stimuli falling in the absolutely refractory period, *i.e.*, in all of our experiments except the above five; hence the lengthening of the premature beat cycle in such experiments could not be due to vagal inhibition.

5—*Conclusions.—It seems, therefore, that the lengthening of the premature beat cycle is directly due to the premature beat. The pacemaker is excited before a beat would normally arise there, and as a consequence an abnormally long interval, the premature beat cycle, elapses before it can again give rise to a beat, i.e., a temporary depression of rhythmicity follows a premature beat.* The length of this premature beat cycle shows that this depression is greater the earlier the premature beat, *i.e.*, the shorter the curtailed cycle.

As judged by the relative lengthening of the premature beat cycle the amount of this depression varies greatly in different experiments, and often it gradually declines throughout an experiment, *e.g.*, fig. 6. In most of such experiments there has been a parallel slowing in the frequency of the beat, which frequently appears to be a consequence of excision of the accelerantes (both or right only). Or it may be that the diminution in lengthening of the premature beat cycle is an indication of a gradual deterioration of the preparation resulting from the initial operative procedures. It does not seem to be related to changes in the ventilation of the preparation, for this has varied both above and below the normal requirements, while any progressive change in the premature beat cycle has always been in the direction of shortening. In some experiments, however, both the premature beat cycle and the frequency of the heart have not appreciably changed over a period of many hours, fig. 7, and in some the frequency has remained constant while there has been a great shortening of the premature beat cycle, fig. 8.

6—*Effect of Accelerantes on Lengthening of the Premature Beat Cycle*—In six experiments single premature beats have been elicited during the acceleration of the heart resulting from tetanic stimulation of the right nervi accelerantes. In fig. 9 the results have been plotted as in fig. 3 and show that the relative lengthening of the premature beat cycle is greater than for the corresponding

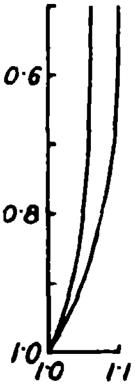

FIG. 6

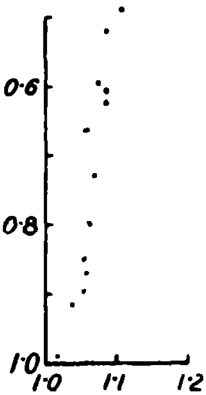

FIG. 7

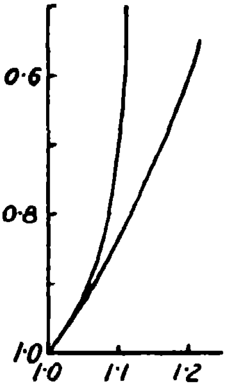

FIG. 8

FIG. 6—Curves of premature beat cycles (abscissæ) plotted against curtailed cycles (ordinates), as in fig. 3, except that abscissæ are plotted only from D onwards. The curve showing the greater lengthening of premature beat cycles was obtained at the beginning of the experiment (length of cycle about 315 σ), the other curve being obtained much later (length of cycle then about 305 σ). FIG. 7—As in fig. 6 but with the individual points plotted to show that in this particular experiment there was no appreciable change in the curve over many hours. FIG. 8—As in fig. 6 in another experiment.

normal rhythm, indicated by the broken line taken from fig. 7 (same experiment). Similar results have been obtained in each of the other five experiments, but in the latter part of one experiment stimulation of the accelerantes failed to alter the relative length of the premature beat cycle, although it still produced a 15% increase in frequency. In the other five experiments the relative lengthening of the premature beat cycle was remarkably constant even though there was a progressive diminution of this lengthening in the absence of accelerantes stimulation. Thus in fig. 10 the observations obtained during accelerantes stimulation showed no gradual change, though without accelerantes there were during the same period the changes shown in fig. 6.

In all these experiments the observations have been made in groups of 10 to 20, each group being taken during a continuous tetanic stimulation of the nervi accelerantes lasting for one to two minutes. A group of normal observations has been made during the period of rest interpolated between each such tetanic stimulation. In three experiments the onset of the acceleration has been so gradual that one or more premature beats have been set up before the full frequency has been attained. In all such experiments the increase in the relative lengthening of the premature beat cycle has lagged behind the acceleration of the rhythm. Thus in fig. 11 the points show the premature beat cycles for those observations which, though soon after the beginning of the accelerantes tetanus, occurred when more than 90% of the maximum change in frequency had taken place. The corresponding curves for later observations in each group (*cf.* fig. 10), and for observations with normal frequency (*cf.* fig. 6) are shown by the broken lines. Conversely if the frequency declines during the latter part of an accelerantes stimulation, there is no parallel decrease in the relative lengthening of the premature beat cycle. For example, a decline of acceleration to half its maximum has not caused any appreciable decrease in the relative lengthening of the premature beat cycle.

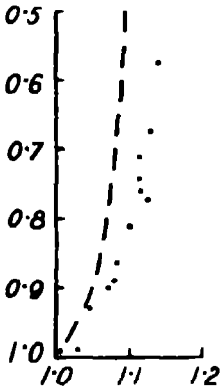

FIG. 9

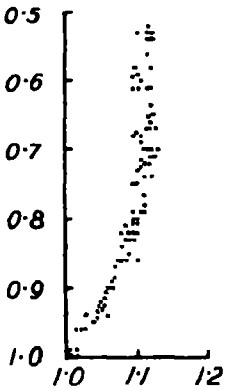

FIG. 10

FIG. 9—Plotting as in fig. 6. The broken line shows the curve for the normal rhythm, cycles 420 σ (see fig. 7 for the points), and the points show observations obtained during shortening of the cycle to 260 σ by tetanic stimulation of the nervi accelerantes.

FIG. 10—As in fig. 9 but in same experiment as fig. 6, the points being obtained during the whole range of the variation of the curve for normal rhythm, the accelerated cycles gradually increasing in duration from 275 σ to 300 σ.

This lack of parallelism between changes in frequency and in relative lengthening of the premature beat cycle is further borne out by the only experiment in which the change of frequency produced by removal of accelerantes tone has been investigated from this point of view. Fig. 12 shows the curve for premature beat cycles before and after excision of both nervi accelerantes. The normal cycle lengthened from 259 σ to 348 σ, but there was practically no change in the relative length of the premature beat cycle. At most there seemed to be a slight decrease in the initial slope of the curve.

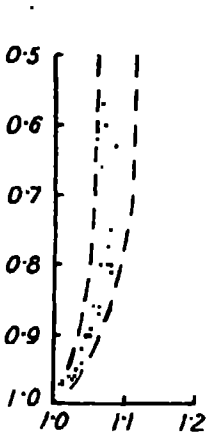

Fig. 11

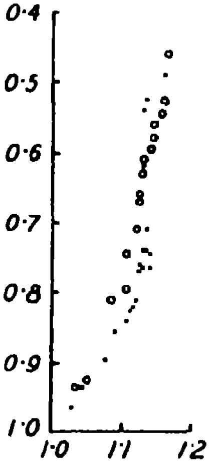

Fig. 12

Fig. 11—Same experiment as fig. 10, the points representing the early observations of the group obtained during each continuous accelerantes tetanus. The broken lines show the curves for the normal rhythm, fig. 6, and for the later observations of each group, fig. 10. Fig. 12—Plotting as in fig. 6. The dots show observations with both nervi accelerantes intact, the circles after their excision with consequent removal of accelerantes tone.

7—*Effect of Vagus on Lengthening of the Premature Beat Cycle*—In eleven experiments single premature beats have been elicited during the slowing of the heart produced by tetanic stimulation of the right vagus. The strength of the submaximal stimulus employed was adjusted so that there was a 10 to 50% lengthening of the cardiac cycle. Only those series of observations have been considered in which the action potentials showed that there was no appreciable shift of pacemaker (p. 318).

In fig. 13 where the results have been plotted as in fig. 3 it will be seen that the relative lengthening of the premature beat cycle is greater than for the corresponding normal rhythm (indicated by the broken line). The slowing of the rhythm together with the usual shortening of the refractory period accounts for some values of curtailed cycles during vagal stimulation being relatively shorter than normal; hence a greater range of the curve can be experimentally determined.

In all the other ten experiments with vagal slowing there was also a greater relative lengthening of the premature beat cycle. In some experiments, *e.g.*, fig. 14, this increase in relative lengthening takes place for all lengths of curtailed cycles, *i.e.*, the curves for normal and vagal slowing deviate from the common point of their origin. In other experiments vagal slowing only causes the curves to deviate with curtailed cycles as short as 0·9 or even shorter, fig. 13. This is especially so where there is with normal rhythms an initially relatively rapid lengthening of the premature beat cycle as the curtailed cycle is shortened.

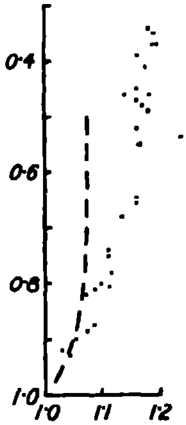

FIG. 13

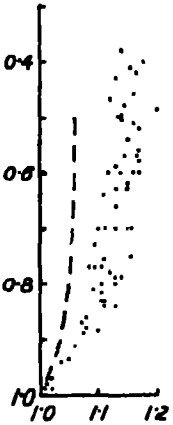

FIG. 14

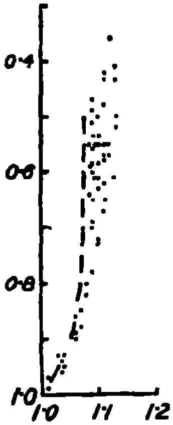

FIG. 15

FIG. 13—As in fig. 9 but the points were obtained during lengthening of the cycle by tetanic stimulation of the vagus from 350 σ to 530 σ. FIG. 14—As in fig. 13, but in another experiment, the cycle being lengthened from 390 σ to 500 σ. FIG. 15—Same experiment as fig. 13, but in fig. 15 the points are from observations in the later part of each group obtained during a continuous vagal tetanus, and in fig. 13 from the earlier part. During the later part of the vagal tetanus the cycle had shortened from 530 σ (see fig. 13) to about 490 σ.

In most experiments about 10 observations have been made during a continuous vagal tetanus in exactly the same way as was done with accelerantes. However, the diminution of vagal effect during stimulation is much more rapid than with accelerantes, and so this method has not proved so satisfactory. In some of these experiments the relative lengthening of the premature beat cycle has diminished much more rapidly than the vagal slowing of the rhythm, *e.g.*, in fig. 15 the points are taken from the latter part of each group of observations and show only about half the increase of relative lengthening obtaining for the early observations of each group, fig. 13, though the average lengthening of the cycle has only decreased from 50 to 40%. In other experiments the changes in the premature beat cycle have been approximately parallel to the changes in the rhythm.

In five experiments an attempt has been made to determine the effect of abolition of vagal tone produced by cutting both vagi. Unfortunately in three the vagal tone was so slight that the increase of frequency was less than 10%. In the two others it was 12% and 15% respectively. In none was there any appreciable change in the relative lengthening of the premature beat cycle. In one of these experiments, that with 12% change, stimulation of the cut right vagus nerve was employed to slow the heart to the rhythm obtaining with tonic action of the vagus. There was then the usual increase in the relative lengthening of the premature beat cycle. Thus it seems that with vagus as well as with accelerantes the effect on rhythm can be dissociated from the effect on the relative lengthening of the premature beat cycle.

Table II—Single Premature Beats

Curtailed cycle	Premature beat cycle	Subsequent cycle	Cycle after subsequent cycle
0·94	1·06	1·00	—
0·94	1·06	0·99	—
0·90	1·075	1·00	—
0·885	1·085	0·99	—
0·87	1·09	1·005	1·00
0·86	1·10	1·00	0·995
0·85	1·10	1·00	—
0·845	1·10	1·00	—
0·81	1·12	1·00	—
0·81	1·125	1·005	1·00
0·775	1·135	1·00	—
0·75	1·15	1·005	—
0·715	1·17	1·015	—
0·71	1·155	1·015	—
0·65	1·155	1·015	—
0·65	1·16	1·02	1·00
0·62	1·175	1·015	—
0·61	1·155	1·03	1·00
0·60	1·18	1·025	1·00
0·58	1·185	1·025	0·99
0·515	1·205	1·04	1·01
0·465	1·23	1·03	0·995

8—*Lengthening of the subsequent Cycle* (*cf.* Lewis and White, 1914; Sansun, 1912)—Table II shows a series of observations from an experiment in which the lengthening of the subsequent cycle was particularly well developed. It will be seen that there is no appreciable lengthening until the curtailed cycle is shorter than 0·75, and that the amount of the lengthening increases as the curtailed cycle is shortened further. Unfortunately the cycle after the subsequent cycle could only be measured in some observations (in the others it was off the plate), but it showed no tendency to differ from the normal cycle. This lengthening of the subsequent cycle cannot be due to stimulation of vagal nerve fibres (p. 334), for it did not occur when the curtailed cycle was long or when the stimulus failed to set up a premature beat owing to its falling in the absolutely refractory period. Moreover, it is probably not due to a temporary displacement of the pacemaker to another rhythm centre, because the action potentials of the beats before and after the lengthened subsequent cycle are indistinguishable from those of normal beats. A possible explanation of the lengthening of the subsequent cycle will be suggested in the next paper (p. 364).

C—*Effect of Two Premature Beats on Rhythm*

The experiments with a single premature beat clearly show that the disturbance in rhythm produced thereby is due to an interference with the rhythmic mechanism of the pacemaker. The lengthening of the premature beat cycle is an expression of the effect produced in this mechanism by the action of the premature beat. One method of analysing this effect is provided by an investigation into the disturbance of rhythm produced by a second premature beat which curtails this lengthened premature beat cycle. A similar method was used when investigating the disturbance of the rhythmic discharge of motoneurones produced by " backfiring " impulses into them (Eccles and Hoff, 1932) and it will be seen that in many respects these two investigations have yielded comparable results.

Fig. 2 is a tracing of one observation from a series in which the curtailed premature beat cycle was 0·913 of a normal cycle, and in fig. 16*a* all the observations of the series have been plotted according to the convention adopted when dealing with the action of single premature beats. In fig. 16*b* the positions of the subsequent beats, *i.e.*, the ends of the second premature beat cycles are shown on a larger scale. Fig. 16*c* shows the observations similarly plotted for curtailed premature beat cycles equal to 0·976 of a normal cycle. Unfortunately there were only three observations in this experiment with fairly short curtailed premature beat cycles (0·665 of a normal cycle). A more complete series is shown in fig. 17 with curtailed premature beat cycles of 0·955, 0·66 and 0·50 of a normal cycle. When the curtailed premature beat cycle is short, the length of the second premature beat cycle is independent of the length of the curtailed normal cycle, and the longer the curtailed premature beat cycle, the more is the length of the second premature beat cycle affected by changes in the length of the curtailed normal cycle. However, when the curtailed premature beat cycle is very short, *e.g.*, less than 0·5, the disturbance of rhythm usually resembles that produced by early-premature-beats. Such

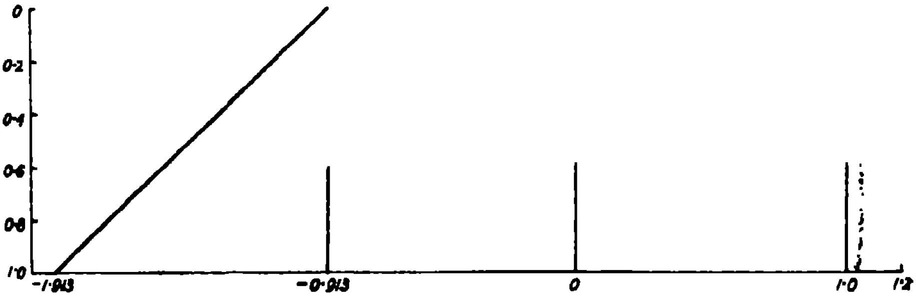

FIG. 16*a*—Observations with two premature beats plotted on the same principles as those for single premature beats in fig. 3. In order that the lengths of the second premature beat cycles should be immediately observable along the abscissæ, the second premature beat has been used as a zero point. The first premature beat occurring in this experiment, 0·913 of a cycle earlier is therefore marked —0·913.

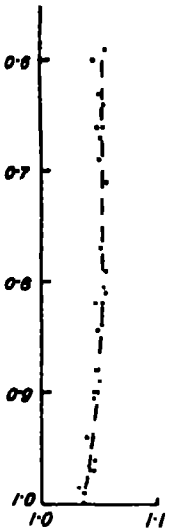

FIG. 16*b*

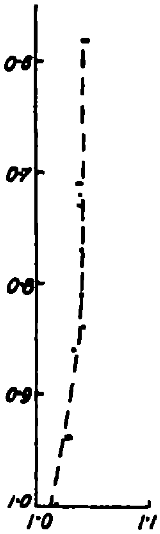

FIG. 16*c*

FIG. 16*b*—The extreme right-hand portion of fig. 16*a*—beyond 1·0—is shown enlarged, the points as before representing the second premature beat cycles plotted against the curtailed normal cycles. The broken line is the curve predicted according to the hypothesis stated on p. 346. FIG. 16*c*—Same experiment as fig. 16*b* but the interval between the two premature beats is 0·976 of a cycle. The broken line again shows the predicted curve.

experiments in which the second premature beat was an early-premature-beat will be dealt with in the next paper.

When investigating the action of two antidromic impulses on the rhythmic discharge of a motoneurone, it was found that the second antidromic impulse was followed by a cycle having a duration corresponding closely with that calculated from the action of a single antidromic impulse on the assumption that with a given rhythm the rhythmic centre of a motoneurone was always in the same condition at any particular interval before its next discharge. A similar assumption has been tried in the present problem, and it has been found that the calculated and experimental values were in close agreement except in those cases where the second premature beat was an early-premature-beat (Eccles and Hoff, 1934*b*).

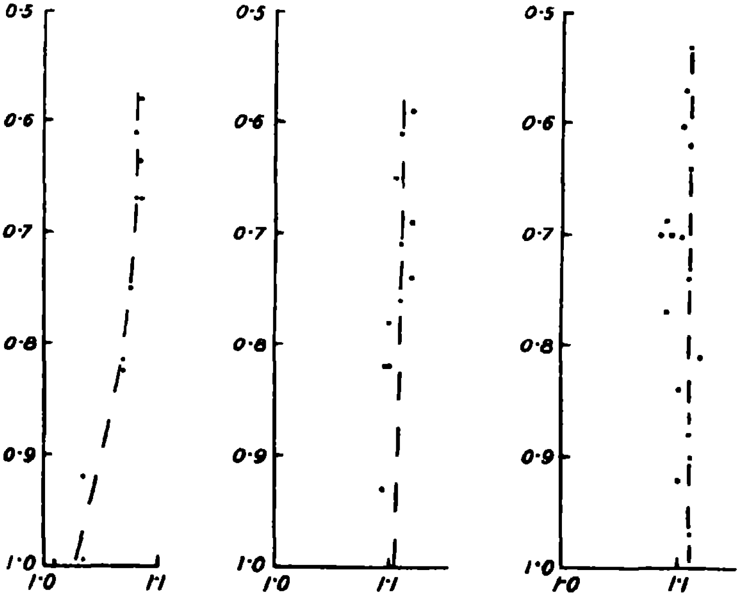

FIG. 17*a* FIG. 17*b* FIG. 17*c*

FIG. 17*a*—As in fig. 16*b* in another experiment with an interval of 0·955 between the two premature beats. The broken line again shows the predicted curve. FIG. 17*b*—Same experiment as fig. 17*a* but interval is 0·66. FIG. 17*c*—Same experiment as fig. 17*a* but interval is 0·50.

In fig. 18 R_1, R_2, R_3, R_4, R_5, R_6 represent the successive normal beats of the pacemaker and the rhythm is interrupted by a premature beat P_1, R_3P_1 being the curtailed normal cycle, and P_1R_4 the premature beat cycle. It has been assumed that at any point X_4 in the latter part of the premature beat cycle the rhythmic centre is in the same condition as at a point X_2 in a normal cycle if X_2R_2 equals X_4R_4. A second premature beat set up at X_4, *e.g.*, P_2 in fig. 19, should therefore be followed by a cycle P_2R_5 equal in length to the premature beat cycle which would follow a single premature beat set up at X_2. For any given interval P_1P_2 between the premature beats (the curtailed premature beat cycle), it will be seen that the interval between P_2 and the hypothetical end R_4 of the premature beat cycle following P_1 will depend on the length of the curtailed normal cycle R_3P_1, for this governs the length of P_1R_4 in the way that has already been discussed (*cf.* p. 330 *et seq.*). And for each interval P_2R_4 it is possible to calculate the expected length of P_2R_5 according to the above assumption. Thus from the curve for single premature beat cycles there can be calculated the expected curve correlating the curtailed normal cycles and the second premature beat cycles for any length of the curtailed premature beat cycle.

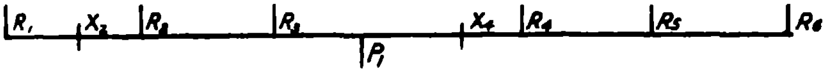

FIG. 18– Schema showing effect of a single premature beat P_1 on the rhythmic beat of the pacemaker. Details in text.

Such calculated curves are drawn as broken lines in figs. 16 and 17 and are seen to be in very close agreement with the points experimentally obtained. Altogether the calculated curve has agreed closely with the experimental observations in twenty-three such series of observations spread over eleven separate experiments. The only exceptions have occurred when the curtailed premature beat cycle has been very short, the second premature beat then functioning as an early-premature-beat (p. 358).

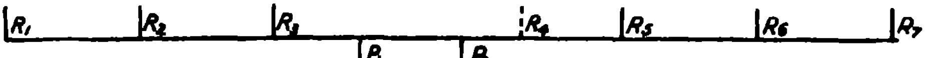

FIG. 19—As in fig. 18 but for premature beats P_1 and P_2.

When the curtailed premature beat cycle has been shorter than 0·6 of a normal cycle, another difficulty has arisen with some calculations. It has been seen in the preceding paper that the refractory period following a premature beat is shorter than normal (p. 321), *i.e.*, after a premature beat it is possible to set up a second premature beat at an interval shorter than that obtaining for the curtailed cycle of the earliest single premature beat. Thus in fig. 20 P_1P_2 is shorter than R_3P_1, P_1 being the earliest possible premature beat after R_3. Moreover, the premature beat cycle after P_1 alone, *i.e.*, P_1R_4, is longer than a normal cycle, so it is clear that P_2R_4 is much longer than any interval at which a single premature beat can be set up *before* the end of a normal cycle. Hence it is not possible to calculate P_2R_5 from the action of a single premature

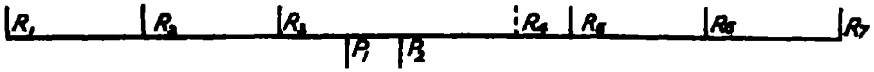

FIG. 20—As in fig. 19 but with a very short interval between the premature beats P_1 and P_2.

beat. However, a sufficiently long extrapolation of the curve for single premature beat cycles is usually easy, for, with short curtailed cycles, it has been seen that the length of the premature beat cycle changes relatively little. The curve drawn in fig. 17*c* is actually calculated in this way.

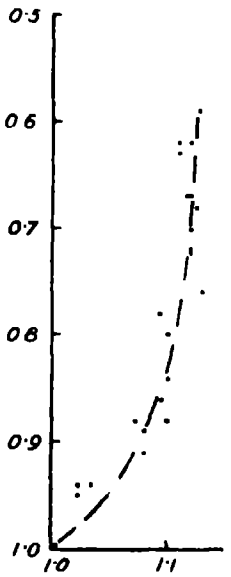

FIG. 21

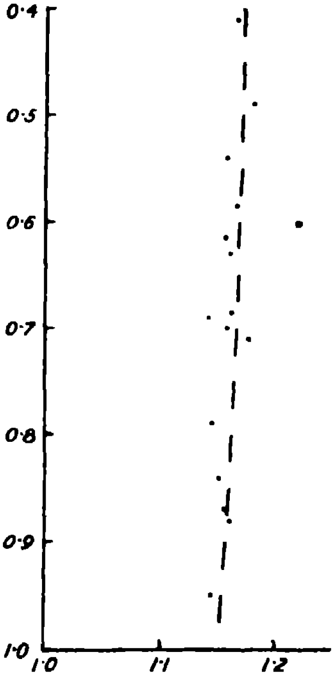

FIG. 22

FIG. 21—As in fig. 16*b* but the observations were made during tetanic stimulation of the nervi accelerantes. The interval between the two premature beats was just equal to the duration of a cycle. As before the broken line shows the theoretical curve. FIG. 22—As in fig. 16*b* but obtained during tetanic stimulation of the vagus, the interval between the two premature beats being 0·47 of a cycle.

Fig. 21 shows that the calculated and observed values are strikingly similar when the rhythm is quickened by tetanic stimulation of the nervi accelerantes.

Good agreement has also been present in those series of observations from the three other experiments in which this investigation has been attempted.

Again fig. 22 shows that there is good agreement when the rhythm is slowed by tetanic stimulation of the vagus nerve. Altogether six such series of observations were obtained in the four experiments in which this investigation was attempted. With the usual degree of vagal slowing (20 to 50% lengthening of cycle) the calculated and observed values have always been similar except when the curtailed premature beat cycle has been so short (less than 0·4) that the second stimulus set up an early-premature-beat.

No deviation has been detected between the calculated and observed values for the second premature beat cycle in those experiments in which the first premature beat cycle was followed by a lengthened subsequent cycle, *e.g.*, Table II, but in such experiments there was a slight lengthening of the subsequent cycle when the curtailed premature beat cycle was short.

The very good agreement between the calculated and experimental values for the second premature beat cycles in all experiments with a sufficiently long curtailed premature beat cycle proves for such conditions the correctness of the assumption underlying the calculation, namely, that *for any given rhythm the rhythmic centre is always in the same condition (so far as the action of premature beats is concerned) at any particular interval before the next spontaneous beat, whether this beat be a normal beat or the subsequent beat terminating a late-premature-beat cycle.* In other words, with any given rhythm the spontaneous beat is always the result of the same temporal course of events in the rhythmic centre so far as is indicated by the action of a premature beat. Now we have seen that the earlier the premature beat, *i.e.*, the shorter the curtailed cycle, the longer is the premature beat cycle. The latter part of the premature beat cycle is, however, similar to the latter part of a normal cycle. *The lengthening of the premature beat cycle must therefore occur during its early part and be due to a delay in setting up the sequence of events which initiates the subsequent beat; and this delay is the longer the shorter the curtailed cycle.*

II—DISCUSSION

A—*Discussion of Hypotheses*

Since a premature beat produces a permanent change in the phase of the rhythm (p. 330), it must act on the rhythmic mechanism itself. Moreover, it has been seen that the lengthening of the premature beat cycle is directly due to this action on the rhythmic mechanism (p. 335). It is therefore clear that the investigation of this premature beat cycle is of fundamental importance for the eventual elucidation of the nature of the processes underlying the rhythmic production of beats.

But a difficulty arises in attempting to formulate a satisfactory provisional hypothesis which would indicate further methods of attack. Most investigators, *e.g.*, Gaskell, Engelmann, and Hering, have postulated two factors as being essentially involved in the production of a rhythmic response (p. 307). One is a form of "inner stimulus" and the other the intrinsic excitability. An hypothesis of this type receives its chief support from the analogy with the processes of excitation by an electrical stimulus.

Now it has been argued (p. 324) that a beat set up by the pacemaker does not arise in the surface membranes of its constituent muscle fibres, but in another mechanism presumably within these muscle fibres. However, though initiated by this inner rhythmic mechanism the beat does not become apparent until it is transmitted to the surface membranes and spreads over them as the characteristic wave of the propagated impulse, for it is only then that it produces an action potential. This inner rhythmic mechanism might possess an "inner stimulus" and an intrinsic excitability, but it is obvious that the extrinsic electrical excitability cannot be regarded as a test for such an intrinsic excitability.

In the related problem of the rhythmic discharge of nerve cells it has been possible directly to determine the time course of the intrinsic excitability (Eccles, 1931; Eccles and Sherrington, 1931, *a*) and it has been found that the rhythmic cycle is often much longer than the time necessary for the complete recovery of this excitability (Eccles and Sherrington, 1931, *b*; Eccles and Hoff, 1932). In such conditions a progressive increase in intrinsic excitability is not a factor concerned in the production of a discharge (beat) so it has been suggested that a discharge is produced solely by a progressive increase of the "inner stimulus" or central excitatory state. When this reaches a certain threshold intensity, a discharge is set up, the central excitatory state is abruptly depressed, and must again be built up to the threshold intensity before the next discharge is produced.

On analogy with this hypothetical description of the mechanism of the rhythmic discharge of nerve cells, the following hypothesis is suggested for the mechanism producing the rhythmic beat of the pacemaker. *In the rhythmic mechanism there is a progressive increase in the "inner stimulus" or excitement until this reaches a threshold intensity. A beat is then set up, the excitement is* *abruptly depressed, and is again slowly built up to threshold at which point it sets up the next beat of the rhythmic series, and so on.*

B—*Consideration of Experimental Results in Terms of Hypothesis*

It has been concluded (p. 346) that the lengthening of the premature beat cycle is due not to a slower building up of excitement during the latter part of the cycle, but to a delay in its early part. Hence it may be concluded that the excitement of the pacemaker is depressed more by a premature beat than by a normal beat. Moreover, this depression is the greater, the earlier the premature beat. The lengthening of the premature beat cycle would be a function of the depth of this depression, and the depth of the depression presumably would be an inverse function of the excitement of the pacemaker at the instant the premature beat was set up*; hence this excitement should be an inverse function of the lengthening of the premature beat cycle. Now as the premature beat falls later and later in the cardiac cycle, the premature beat cycle shortens very little at first; but as evidenced by the outward convexity of the curves, figs. 3, 6. and 7, it shortens with increasing rapidity as the premature beat approaches the time of the next normal beat. On the above reasoning this would indicate that the excitement of the pacemaker increases relatively more slowly at first and then more and more quickly until it attains threshold and so sets up a beat. This abrupt attainment of threshold would appear as a possible factor in determining the extreme regularity of heart rhythm.

Considerable variations in the durations of short curtailed cycles are accompanied by very little change in the corresponding premature beat cycles, hence it is concluded that under such conditions the excitement is depressed almost to a basal level below which it cannot be diminished (*cf.* the basal condition described for the motoneurone, Eccles and Hoff, 1932). Even when the premature beat cycle is greatly curtailed by a second premature beat, the duration of the second premature beat cycle shows that there is very little further diminution of excitement. However, the curtailed premature beat cycle must not be too short, for then the second premature beat is an early-premature-beat and produces the disturbances of rhythm described in the next paper.

The rhythmic responses of nerve cells (Eccles and Hoff, 1932) and of the tension receptors of muscle (Matthews, 1933) have been subjected to an investigation similar to that described in this paper, the rhythm being disturbed by antidromic impulses, which are equivalent in effect to premature beats. In both these investigations the curve correlating the cycles before and after the premature beat has been concave outwards instead of convex as with the pacemaker.

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* This involves the tacit assumption that the premature beat always depresses the excitement by approximately the same amount (*cf.* Eccles and Hoff, 1932).

With the hypothesis in its present undeveloped form no explanation is forthcoming of the wide divergences from experiment to experiment in the relative lengthenings of the premature beat cycles. It has been seen that the gradual changes in any one experiment may be associated with changes in the heart rate, but other factors also appear to be involved. The effect of tetanic stimulation of the accelerantes or vagus can, however, be more profitably considered.

During accelerantes stimulation the relative lengthening of the premature beat cycle is greater than with normal rhythm (p. 336) but, if the lengthenings of duration be measured in generalized time, *i.e.*, in seconds, corresponding premature beat cycles are lengthened by approximately the same amount with normal and accelerated rhythms. In fig. 9, for example, the premature beat cycles with accelerated rhythms are lengthened slightly less than the corresponding cycles with normal rhythms, while, if fig. 10 be compared with fig. 6, the reverse is found to occur. The increase in *relative* lengthening of the premature beat cycle during acceleration seems therefore to be largely due to the shortening of the normal accelerated cycle, the temporal sequence of events during the earlier part of the accelerated premature beat cycle being practically identical with that obtaining during the corresponding premature beat cycles of normal rhythm. It is the latter part of the accelerated premature beat cycle, *i.e.*, the part identical with the normal accelerated cycle, which is shortened. Thus the accelerantes appears to quicken the rhythm by hastening the building up of excitement during the latter part of a cycle. However, the above explanation is incomplete, for it takes no account of the fact that the relative lengthening of the premature beat cycle is not a simple function of the acceleration, *e.g.*, it lags behind the change of rhythm both at the beginning and during the gradual onset of fatigue of an accelerantes tetanus (p. 337).

Similarly the negative chronotropic action of the vagus is not simply related to the increased relative lengthening of the premature beat cycle, for this effect falls off before the effect on the rhythm (p. 339), and with the natural tonic action of the vagus there may be no effect whatever on the lengthening of the premature beat cycle (p. 340). The curves obtained during vagal tetanus have exhibited a progressive lengthening of the premature beat cycle even when relatively short curtailed cycles are further shortened, figs. 13, 14, and 15. This by the above argument (p. 348) would indicate that there was an appreciable increase of excitement relatively much earlier in the cycle than with the normal rhythm. Thus there is a much longer period than normal during which excitement is increasing. This inference fits in well with the conclusion arrived at in a later paper (Brown and Eccles, 1934), that the vagus slows the heart beat not by preventing excitement from accumulating, but by counteracting its action on the rhythmic mechanism so that a greater intensity is necessary to set up a beat; hence the longer period necessary for accumulation. Vagal action appears to produce no delay in the initial stages of the building up of excitement.

SUMMARY

Premature beats have been set up by a stimuli applied directly to the pacemaker and action potentials have been recorded by an amplifier and Matthews oscillograph, one lead being from the pacemaker and the other from the auricle.

Except when premature beats have been set up after very short curtailed cycles (early premature beats considered in the next paper) the cycle next following the single premature beat, *the premature beat cycle*, is lengthened, being the longer the earlier this beat is set up after the preceding normal beat, *i.e.*, the shorter *the curtailed cycle*. The cycle after the premature beat cycle (the subsequent cycle) may be slightly lengthened, but thereafter the normal rhythm is resumed, though with an altered phase for the lengthening of the premature beat cycle (and subsequent cycle) is less than the shortening of the curtailed cycle.

Wenckebach explained the relationship between curtailed and premature beat cycles in terms of the delay occupied by the conduction time to the pacemaker, but this proves inadequate, consequently it is concluded that the lengthening of the premature beat cycle is a direct effect of the premature beat on the rhythmic mechanism of the pacemaker.

Tetanic stimulation of the nervi accelerantes or of the vagus produces a greater relative lengthening of the premature beat cycle, but with neither stimulation does this lengthening appear to be simply related to the alterations in the length of the normal cycle.

The disturbance of rhythm produced by two premature beats has been investigated when the second curtails the first premature beat cycle to a varying degree but not to such an extent that it is an early-premature-beat. The

second premature beat cycle has been plotted against the curtailed normal cycle, and it has been found that the experimental results closely agree with the values calculated from the curve for single premature beats, if the assumption be made that for any given rhythm the rhythmic centre is always in the same condition of functional excitement at any particular interval before the next spontaneous beat.

In a provisional hypothesis it is tentatively suggested that there is in the rhythmic centre a condition called *excitement*, which gradually increases throughout the cycle and sets up a beat when it attains a certain threshold intensity. It is immediately depressed, and must again be built up to threshold before the next beat can be set up. The results are discussed in terms of this conception, but no quantitative development of the hypothesis has been possible.

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The Rhythm of the Heart Beat. III—*Disturbances of Rhythm produced by Early Premature Beats*

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In the preceding paper it was shown that the disturbance of rhythm produced by a premature beat introduced in the latter part of a normal cycle or of a premature beat cycle is a comparatively simple and regular one: the cycle following the premature beat so introduced is lengthened by an amount that varies with the degree of curtailment of the preceding cycle. The subsequent cycle is normal, or, when the shortening of the curtailed normal cycle is more marked, it may be slightly lengthened. This paper will deal with the disturbances of rhythm produced by a premature beat introduced earlier in a normal cycle or a premature beat cycle; they are more complex and less regular. Responses of this type have previously been observed by Drury and Brow (1926), who called them re-entrant beats, and by Miki and Rothberger (1922), but no attempt has been made to elucidate the mechanism of their production. The method used was that described in the previous paper, p. 308.

I—Experimental Results

A—*Disturbance of Rhythm produced by an Early-premature-beat Curtailing a Normal Cycle* (*Early-subsequent-beats*)

The whole gamut of the effects of premature beats on rhythm is illustrated by the series of observations of Table I, which is similar in construction to Table I of the previous paper. The upper part of the table shows the lengthening of the premature beat cycle when the curtailed normal cycle is long (late-premature-beats). As the curtailed normal cycle shortens to less than 0·50, there also seems to be some lengthening of the subsequent cycle (*cf.* Tables I and II of the previous paper). The lower part of the table shows that with early-premature-beats (curtailed normal cycle 0·345 or less) the premature beat cycle is greatly shortened by the appearance of an *early-subsequent-beat*, while the subsequent cycle is usually lengthened. The lowest two observations show that, when the curtailed normal cycle is very short indeed, the premature beat cycle and even the subsequent cycle may also be exceedingly short. Fig. 1 is a tracing of an observation in which an early-subsequent-beat appeared.

Table I—Single premature beats

Curtailed normal cycle	Premature beat cycle	Subsequent cycle	
0·98	1·02	1·01	Late-premature-beats
0·91	1·085	0·99	
0·87	1·125	0·995	
0·82	1·13	0·995	
0·75	1·16	1·00	
0·74	1·17	0·995	
0·67	1·18	0·99	
0·63	1·185	1·005	
0·60	1·20	1·01	
0·60	1·20	1·02	
0·56	1·23	1·00	
0·50	1·20	1·00	
0·50	1·21	1·00	
0·49	1·205	1·025	
0·48	1·20	1·00	
0·43	1·19	1·02	
0·42	1·205	1·02	
0·41	1·225	1·02	
0·38	1·22	1·02	
0·32	1·22	1·02	
0·345	0·74	1·19	Early-premature-beats
0·34	0·665	1·07	
0·34	0·66	1·20	
0·34	0·64	1·185	
0·32	0·57	1·215	
0·32	0·61	1·19	
0·31	0·68	1·27	
0·275	0·70	1·27	
0·26	0·84	0·99	
0·175	0·20	1·27	
0·175	0·15	0·26	(next cycle 1·08)

Early-subsequent-beats have only arisen after single premature beats in about one-third of our experiments, but in all such experiments (10) there has been a critical duration of curtailed normal cycle below which early-subsequent-beats have always been set up. This is the basis of the subdivision of premature beats into late-premature-beats producing the disturbances of rhythm dealt with in the previous paper, and early-premature-beats, which are characterized by the setting up of early-subsequent-beats. Usually there has been a slight lengthening of subsequent cycle following those late-premature-beats which are somewhat longer than this critical duration, and in some this lengthening has been much more obvious than in Table I, but in a few, *e.g.*, Table II, this transitional condition does not seem to be present.

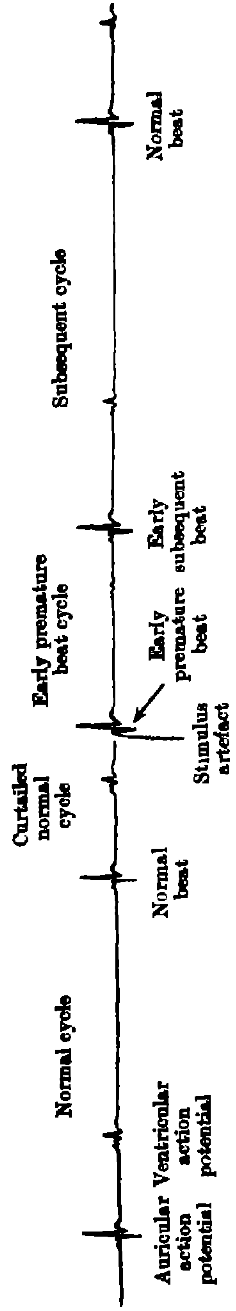

FIG. 1—A tracing of the action potential record in an observation in which an early-subsequent-beat was set up.

The following generalizations concerning early-subsequent-beats have been based on a close examination of the records of the above 10 experiments.

(1) The critical degree of shortening of the curtailed normal cycle necessary for the appearance of early-subsequent-beats varies from experiment to experiment and it bears no regular relation to the absolutely refractory period. If the latter were the longer, then early-subsequent-beats could not arise, a condition obtaining in two-thirds of our experiments with single premature beats.

(2) The experiments in which early-subsequent-beats appeared were mostly those in which late-premature-beats were followed by considerably lengthened premature beat cycles (1·20 or more).

(3) In six of the 10 experiments early-subsequent-beats only occurred during tetanic stimulation of the vagus. In five this appeared to be associated with a concomitant shortening of refractory period, for the critical duration of the curtailed cycle was usually shorter than the refractory period obtaining in the absence of vagal stimulation. In only one experiment did the vagus definitely have no effect on the occurrence of early-subsequent-beats, and in this it also had no effect on the duration of the refractory period. The vagus stimulation was at times strong enough to displace the pacemaker to some other part of the sino-auricular node, as shown by change in action potentials, but it never displaced the pacemaker to the auriculo-ventricular node. Tetanic stimulation of the nervi accelerantes never had any appreciable effect on the occurrence of early-subsequent-beats.

(4) There has been no constant relationship between the durations of curtailed cycles and of the corresponding premature

beat cycles terminated by early-subsequent-beats. Usually there has been a tendency for shorter curtailed cycles to be followed by shorter premature beat cycles (*cf.* Table II).

(5) In two experiments the refractory period of the pacemaker after an early-premature-beat was determined and was found to be similar to that obtaining after a late-premature-beat, whose curtailed cycle was just longer than the critical value. A similar duration was also found for the refractory period following an early-subsequent-beat. It seems, therefore, that neither early-premature-beats nor early-subsequent-beats can be distinguished from late-premature-beats by the duration of the refractory state which follows them.

Table II—Single premature beats—only some of the observations with late premature beats are shown

Curtailed normal cycle	Premature beat cycle	Subsequent cycle	
0·97	1·025	1·00	Late-premature-beats
0·94	1·04	1·00	
0·885	1·07	1·00	
0·865	1·10	1·00	
0·85	1·13	1·005	
0·72	1·17	1·00	
0·69	1·145	1·01	
0·60	1·16	1·005	
0·56	1·175	0·995	
0·47	1·20	1·00	
0·47	0·96	1·175	Early-premature-beats
0·45	0·70	1·16	
0·45	0·45	1·02	
0·44	0·50	1·12	
0·44	0·48	1·055	
0·44	0·46	1·065	
0·42	0·56	1·18	
0·41	0·345	?	(off Plate)

(6) The action potentials of early-subsequent-beats were sometimes indistinguishable from normal beats (fig. 1, *cf.* Drury and Brow, 1926). More often they differed from normal, indicating that the early-subsequent-beats had arisen in parts of the sino-auricular node some distance from the pacemaker, but in no case did early-subsequent-beats appear to have arisen in the auriculo-ventricular node.

(7) In five experiments early-premature-beats with very short curtailed cycles were followed by a rapid irregular series of beats (*cf.* the lowest observation of Table I). This is apparently the condition described by Lewis, Drury, and Bulger (1921) as " rapid re-excitation."

Theories of the Production of Early Spontaneous Beats—1. Kisch (1921) working on dying rabbits' hearts, and Drury and Brow (1926) using dogs' hearts, observed that after an auricular extra-systole (premature beat) the subsequent beat sometimes occurred at exactly the moment when the next spontaneous beat would have been due, had no extra-systole been produced *i.e.*, the combined lengths of the curtailed normal cycle plus the premature beat cycle equalled one normal cycle. They supposed that in these cases the premature beat had not disturbed the rhythm of the pacemaker at all, and they called it an "interpolated extra-systole." Drury and Brow explained this by assuming that the interpolated extra-systole was set up early in the cycle while the pacemaker was still refractory.

The present records contain a few examples where the sum of the curtailed normal cycle and the premature beat cycle has equalled one normal cycle, and these may possibly be susceptible of Drury and Brow's explanation. In the great majority of examples, however, as Tables I and II show, the sum is either greater or less than one normal cycle, and Drury and Brow also mention such discrepant examples in their records. Their explanation, therefore, cannot hold for the appearance of early-subsequent-beats in general, and it is not unlikely that the occasional occurence of a subsequent beat at the moment when it would have arisen in an undisturbed pacemaker is merely a chance happening.

2. An analogous explanation, however, suggests itself, and requires closer examination. It is generally accepted that the sino-auricular node contains many potential pacemakers as well as one actual pacemaker. These are all rhythmic centres, varying somewhat in their intrinsic rate of discharge. The actual pacemaker is the one with the fastest rate, and every impulse arising in it discharges all the others as well; hence none of them succeeds in initiating an impulse, unless the actual pacemaker is in some way depressed. It is conceivable that one of these subsidiary centres might have a longer refractory period than the pacemaker (as well as a slower rhythm), and that an early-premature-beat might fall inside this refractory period, although outside the refractory period of the pacemaker. In that case, the early-premature-beat would discharge the pacemaker, but not the subsidiary centre, and the latter might then give rise to an early-subsequent-beat.

This conception cannot explain those cases where the sum of the curtailed normal cycle and the premature beat cycle is equal to or less than one normal cycle, for it regards all subsidiary centres where the subsequent beat might possibly arise as possessing a slower rhythm, *i.e.*, longer cycles, than the actual normal pacemaker. It can be entertained only in respect of those examples where the sum is greater than one normal cycle. In these cases it can be tested by the method developed in the preceding paper (p. 343); for, if the early-subsequent-beat arises elsewhere than in the normal pacemaker, it will affect the pacemaker in the same way as a premature beat induced by electrical stimulation, and can be treated as a second premature beat curtailing the first premature beat cycle.

In fig. 2, R_1, R_2, R_3 represent normal beats arising in the pacemaker, and being conducted to and exciting a subsidiary rhythmic centre C, at C_1, C_2, C_3. P is a premature beat set up by electrical stimulation, which excites the pacemaker, but not the subsidiary centre C on account of its longer refractory period. C then gives rise to the early-subsequent-beat C_4, which is conducted back to the pacemaker and excites it at R_4, thus cutting short the premature beat cycle which would otherwise have terminated at X. In any given experiment the point X is predictable from the curve relating the lengthening of premature beat cycles to the length of the preceding curtailed normal cycles (see previous paper, p. 332); a short extrapolation beyond the critical point where early-subsequent-beats appear is necessary, but this is justified, for the purpose of this argument, by the assumption from which the argument proceeds, namely, that the early-subsequent-beat does not arise in the pacemaker. X having been determined in this way, R_4 is treated as a second premature beat introduced in the first premature beat cycle PX, and the expected length of the second premature beat cycle R_4R_5 is then determined from the above-mentioned curve. This expected length has been compared with the actual length of the cycle after the early-subsequent-beat in a large number of examples. No satisfactory agreement was found (*cf.* Tables I and II); the actual length was often much shorter than the expected length, and the discrepancy would have been increased had allowance been made for the conducting time C_4R_4.

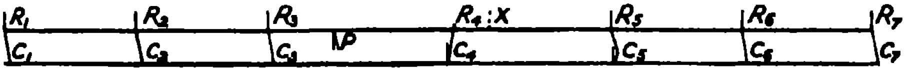

FIG. 2—Schema illustrating a possible explanation of an early-subsequent-beat appearing after a single premature beat. Details in text.

Delay in the restoration of pacemaking function from C to R will not explain the discrepancy, for the cycles after the subsequent cycle were of normal duration except in those cases where "rapid re-excitation" was produced (lowest record of Table I).

We must therefore abandon the conception that early-subsequent-beats arise in subsidiary rhythmic centres outside the pacemaker which itself is behaving in the same way as it does with late-premature-beats. This explanation could hold for some observations of Tables I and II, but it completely fails as a general explanation of early-subsequent-beats.

An alternative explanation of the origin of early-subsequent-beats will be suggested in the discussion at the end of this paper.

B—*Disturbance of Rhythm produced by an Early-premature-beat Curtailing a Premature Beat Cycle*

In all the experiments in which these observations were made, the disturbance produced by single premature beats had also been studied, and it was known whether it was possible in each case to set up early-premature-beats, *i.e.*, to obtain early-subsequent-beats.

Table III—Two premature beats

Curtailed normal cycle	Curtailed premature beat cycle	Second premature beat cycle	Subsequent cycle
0·845	0·485	1·155	1·05
0·83	0·485	1·145	1·045
0·81	0·45	1·145	1·035
0·785	0·485	1·15	1·04
0·78	0·45	0·575	1·12
0·75	0·45	0·58	1·10
0·70	0·45	0·615	1·10
0·61	0·45	0·654	1·11
0·595	0·45	0·635	1·13
0·54	0·45	0·73	1·13
0·53	0·45	0·99	1·045
0·525	0·45	1·015	1·04
0·51	0·45	1·015	1·025
0·50	0·45	1·025	1·025
0·485	0·45	0·905	1·035
0·48	0·45	0·94	1·015

I—*Disturbance Produced by an Early-premature-beat Curtailing a* Late-*premature-beat Cycle*— This is exemplified by Table III which is taken from an experiment in which single premature beats were never followed by early-subsequent-beats, *i.e.*, early-premature-beats were unobtainable; all the first premature beats are therefore "late." The curtailed normal cycles in the first column range from 0·48 to 0·845; when the curtailed normal cycle was longer than 0·845, however, the refractory period following the first premature beat was so long that the second stimulus failed to produce a beat. The upper part of the table is completed by introducing three observations with a slightly longer interval (0·485) between the premature beats. As the curtailed normal cycle becomes progressively shorter, the second premature beat cycle and the subsequent cycle undergo changes which show two abrupt transitions, dividing the table into three sections.

In the uppermost section, the second premature beat cycle is longer than a normal cycle, and the subsequent cycle is slightly lengthened by an amount comparable with that observed after the earliest of single late-premature-beats (p. 340). The second premature beat is therefore behaving, here, as a late-premature-beat, so the disturbance of rhythm is the same as with two late-premature-beats in the preceding paper (p. 341).

Then comes a sharp " upper transition " to the middle section of the table. The second premature beat cycle becomes much shorter, while the subsequent cycle becomes more markedly lengthened. The second premature beat is now behaving like the single early-premature-beat described above in this paper, being followed by an early-subsequent-beat and a lengthened subsequent cycle.

There follows a second sharp " lower transition " to the lower section of the table, in which the second premature beat cycle lengthens to the neighbourhood of 1·0, and the subsequent cycle is still lengthened but less than in the middle section.

In five experiments results essentially similar to those of Table III have been obtained and the following general statements may be made :—

(1) In the upper section, the lengthening of the second premature beat cycle usually agreed with the amount predicted by the method used in the previous paper (p. 343). In a few cases the observed values were longer than the predicted ones and when present these unexpectedly long observed values were always just above the upper transition, *i.e.*, as the curtailed normal cycle was shortened, they appeared as a transitional condition preceding the stage of early-subsequent-beats.

(2) The upper transition was always sharp.

(3) The short second premature beat cycles in the middle section sometimes showed a progressive lengthening as the curtailed normal cycle became shorter. This is shown in Table III but was not constant.

(4) The lower transition is sometimes distinct and sometimes blurred.

(5) The lengthening of the subsequent cycles in the middle and lower sections was usually less than that predicted by the method described on p. 357, starting from the conception that the early-subsequent-beat arises from a centre

outside the pacemaker, which is itself behaving as with late-premature-beats. That conception therefore will not do for a general explanation of early-subsequent-beats produced by an early-premature-beat curtailing late-premature-beat cycles. The above conception is also ruled out by the fact that the curtailed first premature beat cycle plus the second premature beat cycle is often *less* than the duration of the first premature beat cycle when it was not curtailed. For example, with the upper observations of the middle section of Table III, the first premature beat cycle alone would have had a duration greater than 1·10, but the sum of columns 2 and 3 is much less than this.

(6) The action potentials of early-subsequent-beats often resembled in shape the action potentials of normal beats.

In these experiments further series of observations were made with certain conditions altered, and they yielded the following results.

(i) With longer intervals between the two premature beats, *i.e.*, less curtailment of the first premature beat cycle, the section of the table above the first transition became extended at the expense of the lower. Thus in Table IV the upper transition occurred, *i.e.*, early-subsequent-beats appeared, only when the curtailed normal cycle had become as short as 0·52, and the lower transition failed to appear. With still longer intervals early-subsequent-beats did not arise, the disturbance of rhythm being the same as that discussed in the previous paper (p. 341).

Table IV—Two premature beats

Curtailed normal cycle	Curtailed premature beat cycle	Second premature beat cycle	Subsequent cycle
0·955	0·515	1·135	1·025
0·945	0·515	1·12	1·025
0·885	0·515	1·13	1·045
0·88	0·515	1·13	1·035
0·85	0·515	1·115	1·03
0·84	0·515	1·12	1·045
0·815	0·515	1·12	1·04
0·79	0·515	1·105	1·03
0·79	0·515	1·10	1·035
0·73	0·515	1·095	1·035
0·71	0·515	1·09	1·03
0·625	0·515	1·09	1·03
0·585	0·515	1·10	1·03
0·565	0·515	1·085	1·025
0·52	0·515	0·87	1·145
0·51	0·515	0·92	1·13
0·485	0·515	0·81	1·11
0·485	0·515	0·815	1·125

(ii) With intervals between the two premature beats progressively shorter than that used for series like Table III, *i.e.*, with greater curtailment of the first premature beat cycle, the curtailed normal cycle had of course to be progressively shorter in order that there should be a sufficient shortening of refractory period (p. 321) to allow the second premature beat to be set up. This downward encroachment of the upper limit of the series did not appreciably affect the transitions, until it abolished them by passing below them.

(iii) Tetanic vagal stimulation and tetanic accelerantes stimulation did not alter any significant features of the result except that vagal stimulation made it possible to set up single early-premature-beats in experiments where without vagal stimulation they were unobtainable (*cf.* p. 354); these cases will be considered in the next section.

Besides the five experiments discussed above, six experiments gave observations similar to the series with long intervals between the two premature beats, Table IV. Series similar to Table III were not obtained on account of the increased difficulty in eliciting early-subsequent-beats. Such experiments represent a condition transitional between the above five experiments and our single experiment in which two premature beats failed to elicit early-subsequent-beats even at the shortest interval at which they could be set up.

II—*Disturbance of Rhythm Produced by a Premature Beat Curtailing an Early-premature-beat Cycle*—It has been seen (p. 353) that early-premature-beats, *i.e.*, single premature beats that are followed by early-subsequent-beats, are obtainable only in certain experiments. The effect of curtailing an early-premature-beat cycle by a second premature beat has only been studied in three experiments. Table V shows one series obtained during tetanic vagal stimulation. For the conditions of this experiment the critical shortening of the curtained normal cycle separating early- from late-premature-beats was about 0·365 (0·34–0·39). In each of the lowest two observations of Table V, the first premature beat was therefore an early-premature-beat. Its cycle was curtailed in turn by a second premature beat, and the second premature beat cycle is seen to be longer than a normal cycle, but it is a little shorter than the duration predicted from the effect of single late-premature-beats. The upper part of the table is also interesting, for the second premature beat cycle is short, *i.e.*, early-subsequent-beats occur, with all lengths of the curtailed normal cycle. This, of course, is to be expected because the interval between the two premature beats (0·30) is shorter than the critical duration of the curtailed cycle (about 0·365) separating early- from late-premature-beats. The whole upper part of Table V would therefore correspond to the lowest sections of Tables I and II, and presumably to the middle section of

Table III. In some other series there has also seemed to be a transition in the lengths of the second premature beat cycle according as to whether the first premature beat cycle was "early" or "late," but in many no such transition has appeared. A systematic study of this section is needed before any conclusions can be drawn.

Table V—Two premature beats

Curtailed normal cycle	Curtailed premature beat cycle	Second premature beat cycle	Subsequent cycle
1·00	0·30	0·875	1·065
0·98	0·30	0·85	1·005
0·975	0·30	0·82	1·025
0·88	0·30	0·88	1·035
0·88	0·30	0·85	1·00
0·79	0·30	0·86	1·00
0·695	0·30	0·925	1·04
0·64	0·30	0·915	1·00
0·62	0·30	0·925	1·00
0·585	0·30	0·93	0·96
0·57	0·30	0·96	1·06
0·555	0·30	0·93	1·025
0·525	0·30	0·95	1·025
0·475	0·30	0·96	1·045
0·47	0·30	1·01	1·045
0·45	0·30	0·93	1·01
0·415	0·30	0·94	0·99
0·325	0·30	1·165	0·025
0·305	0·30	1·13	1·00

II—DISCUSSION

The action potentials of many early-subsequent-beats so closely resemble the normal action potential that such beats must have originated in the very near neighbourhood of the normal pacemaker, if not actually in it. Doubtless some early-subsequent-beats have arisen from rhythmic centres other than the pacemaker, but even in many of these cases the lengths of the subsequent cycles show that the pacemaker is not behaving as with late-premature-beats (pp. 357-360). Moreover, the normal pacemaker is probably distinguished from other rhythmic centres in the sino-auricular node solely by its slightly faster intrinsic rhythm. Hence it is justifiable as a first approximation to consider all early-subsequent-beats as arising in a single rhythmic centre—the pacemaker.

In contrast to the sharp transition between the disturbances of rhythm produced by early- and late-premature-beats, there is a gradual transition between the normal rhythm and the disturbance of rhythm produced by late-premature-beats, all intermediate stages existing as a premature beat falls later and later until it just anticipates the setting up of a normal beat (p. 333). Moreover, by assuming that the latter parts of late-premature-beat cycles are the same as those of normal cycles, it is possible to calculate the durations of the second premature beat cycles when late-premature-beats curtail late-premature-beat cycles (p. 344), but this is not possible with early-premature-beats. It seems, therefore, that normal beats and late-premature-beats form a class apart from early-premature-beats.

Hypothetical Conception of the Action of the Pacemaker—It is assumed that the rhythmic mechanism of the pacemaker has a certain extensity, its diverse parts being co-ordinated into a functional unit by some system of integration. A beat is set up when the integrated excitement of the whole rhythmic mechanism attains a certain threshold intensity, and an immediate depression of excitement results. With the exception of the concept of integration this hypothesis has already been developed as an explanation of normal rhythm and of the disturbances of rhythm produced by late-premature-beats (see previous paper, p. 347, for a full statement). In such cases no consideration of integration was necessary, for all parts of the rhythmic mechanism are assumed to be equally involved by normal beats and late-premature-beats.

With early-premature-beats, however, it is assumed that only a fraction of the rhythmic mechanism of the pacemaker is fired off, the remainder of the pacemaker being refractory. In the unexcited fraction there would be no depression of the excitement, which would, on the contrary, continue to increase; and so for the next beat this fraction would provide much more than its normal share of the threshold intensity of the integrated excitement. As a consequence threshold intensity would be attained and a subsequent beat set up earlier than after a late-premature-beat. Such an early-subsequent-beat would probably be followed by a subsequent cycle shorter than that predicted from the effect of late premature beats (p. 357), for after this beat the previously unexcited fraction presumably would still have an excitement greater than it would have after a normal beat,* and so it would again contribute more than its normal share of the integrated excitement. Now, for the subsequent cycle, the observed discrepancy has almost always been a shortening (p. 357), hence in this respect the explanation fits in well with experiment.

* The previously excited fraction would have an excitement less than after a normal beat, but this depression would be limited by the basal level below which excitement cannot be reduced (p. 348).

If the unexcited part forms only a small fraction of the rhythmic mechanism, a great increase in the intensity of its excitement will contribute relatively little to the integrated excitement, and so will produce only a small deviation from the normal response times. This would perhaps be the explanation of those observations with single late-premature-beats in which the subsequent cycle was slightly longer than normal (p. 340). In such cases the premature beat cycle itself is probably terminated by a slightly early subsequent beat, but whether this is so cannot be determined as there is, of course, no standard by which to judge it.

A small fraction of the integrated excitement would also be provided by a part of the rhythmic mechanism not closely linked with other parts. The closer this linkage the more would the integration approximate to a simple summation of the individual excitements. It is conceivable that all degrees of this linkage could exist between the different parts of a rhythmic centre, and in extreme cases of dissociation the diverse parts could be practically autonomous. The sharp transitions which occur in series of observations on early spontaneous beats (Tables I, II, III, and IV) presumably depend on large parts of the rhythmic mechanism functioning as an indivisible unit.

The refractoriness of the part of the pacemaker not fired off by early-premature-beats could arise in two ways :—

1. By some of the constituent fibres of the pacemaker being in an absolutely refractory condition at the time of the premature beat, so that the impulse could only pass over part of the pacemaker and hence only fire off part of the rhythmic mechanism.

2. By part of the rhythmic mechanism itself being in an absolutely refractory condition, so that it could not be fired off by the propagated impulse of the premature beat, even though this traversed the whole centre.

On the available evidence it is not possible to decide between these alternative explanations, but the following arguments show that the second explanation is not so improbable as it might at first seem.

(i) In a previous paper (p. 324) experimental evidence indicated that the rhythmic mechanism is not situated in the surface membranes of the fibres constituting the pacemaker, and it was suggested that the rhythmic mechanism might be analogous to the inner contractile mechanism of skeletal muscle fibres, which has been stimulated by Gelfan and Gerard (1930) without setting up a propagated impulse in the surface membranes (Gelfan and Bishop, 1932). Moreover, excitation of this inner contractile mechanism may be propagated without giving rise to any detectable action potential (Gelfan and Bishop, 1933). However, it has not yet been ascertained if this inner contractile mechanism has a characteristic refractory period analogous to that postulated in the above second alternative, though Gelfan's observation that stimulating currents of long duration may evoke twitch-like submaximal responses (1933) seems indirect evidence for the existence of a refractory period of the inner contractile mechanism. If the rhythmic mechanism is analogous to the inner contractile mechanism, it should be capable of direct excitation without setting up a propagated impulse. However, the relative diffuseness of the stimulating current in the present experiments would prevent this from being observed.

(ii) According to the first alternative the propagated impulse of the early-premature-beat does not traverse some parts of the pacemaker, so these parts should not become refractory. But no such partial refractoriness has been found after early-premature-beats (p. 355)—a state of affairs which would be expected according to the second alternative.

(iii) Early-premature-beats are set up most readily when the refractory period following a propagated impulse is shortened either by stimulating the vagus (p. 354) or by a preceding premature beat (p. 358). This would be expected from the explanation of early-premature-beats in terms of a refractory condition of the pacemaker, if this refractory condition were less shortened than the refractory period for the propagated impulse, *i.e.*, two separate refractory periods in separate structures are envisaged.

The above discussions show that the " fractionation " hypothesis provides a plausible explanation of the usual disturbances of rhythm produced by early-premature-beats, but in its present form it fails to explain the following experimental results :—

(*a*) In a few experiments there was a transitional condition between late- and early-premature-beats during which the second premature beat cycle was much longer than the value predicted for late-premature-beats (p. 359). This condition may be explained when the lengthening of the late-premature-beat cycle (p. 348) is better understood.

(*b*) In some experiments the early-premature-beat cycle has been so short that the sum of the curtailed normal cycle and the premature beat cycle has been less than a normal cycle (Tables I and II), *i.e.*, owing to the early-premature-beat the rhythmic mechanism has set up a beat (the early-subsequent-beat) sooner than it otherwise would have done (*cf.* Drury and Brow, 1926). A similar condition has been observed when early-premature-beats curtail late-premature-beat cycles, the sum of the curtailed premature beat cycle and the second premature beat cycle then being less than the first premature beat cycle alone (*cf.* p. 360, and Table III). In both these cycles the early-premature-beat has removed some factor which normally "inhibits" the production of a beat by the rhythmic mechanism. This "inhibitory" factor is not due to the vagus, for it is observed in the absence of vagal action, and vagal inhibition is not affected by a normal beat or a premature beat (Brown and Eccles, 1934). Doubtless even when early-premature-beat cycles are not so short as those just considered, the removal of this "inhibitory" factor plays some part at least in shortening such cycles, *i.e.*, in setting up the usual type of early-subsequent-beats. In fact, it is conceivable that removal of this "inhibitory" factor constitutes the sole condition determining the setting up of early-subsequent-beats.

Such an hypothesis would also seem to involve a fractionation of the rhythm centre by an early-premature-beat.

Conclusions

An attempt has been made to explain the production of early-subsequent-beats by assuming that early-premature-beats fire off only a fraction of the rhythmic mechanism of the pacemaker. Most of the disturbances of rhythm produced by early-premature-beats can be explained by this fractionation hypothesis, but such explanations as we have attempted are intended merely to be illustrative of the way in which such an hypothesis could be developed. The present hypothesis will be justified if it directs attention to the integrative character of the functional processes of a rhythmic centre. As yet we have no concepts of the processes subserving such an integration between diverse parts of a living tissue, but a similar condition forms the basis of summation of central excitatory state existing in different parts of a nerve cell (Eccles and Sherrington, 1931, p. 604), and in other functional activities the parts of a living cell must be closely bound together by unknown processes of association.

We wish to express our gratitude to Dr. R. S. Aitken for his generous and valuable help in rewriting these three papers. We also wish to thank the Christopher Welch Trustees for defraying the expense of the photographic plates, and one of us (J. C. E.) has to thank the Medical Research Council for a personal grant.

III—SUMMARY

As a premature beat is set up with a progressively shorter curtailed cycle, the disturbance of rhythm produced by it passes through an abrupt transition from the lengthening of the premature beat cycle (characteristic of late-premature-beats) to a shortening of the premature beat cycle (characteristic of early-premature-beats). The early-subsequent-beats which terminate early-premature-beat cycles are in turn followed by subsequent cycles which are usually longer than normal cycles, but thereafter the normal rhythm is restored.

An early subsequent beat cannot be explained simply as the response of a rhythmic centre which, owing to its refractory period, was not fired off by the early-premature-beat, the pacemaker itself behaving as with late-premature-beats. Many early-subsequent-beats actually appear to arise in the pacemaker, so it is clear that the disturbance of rhythm produced by early-premature-beats directly involves the pacemaker.

Early-subsequent-beats have been most frequently set up when a late-premature-beat cycle is greatly curtailed by a second premature beat, and for such conditions the effects of variations in lengths of the curtailed normal cycle and of the curtailed premature beat cycle have been investigated.

In explaining the disturbances of rhythm produced by early-premature-beats it is assumed that there is an integration of the excitement present in the diverse parts of the pacemaker, and a beat is set up when this integrated excitement attains threshold intensity, there being then an immediate depression of excitement. Further, it is assumed that normal beats and late-premature-beats fire off the whole rhythmic mechanism of the pacemaker while early-premature-beats only fire off part, the remainder being refractory. In this unexcited fraction excitement is not depressed, but continues to increase, and so a subsequent beat is set up earlier than after a late-premature-beat.

This provisional hypothesis is discussed in relation to the experimental evidence and it is found to explain many of the disturbances of rhythm produced by early-premature-beats, but certain observations have presented difficulties.

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The Oxygen to Iron Ratio of Oxychlorocruorin and the Total Quantity of Oxygen carried by the Pigment in Spirographis.

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1. *The Ratio of Oxygen to Iron in Oxychlorocruorin*

There exist four respiratory pigments capable of uniting with oxygen in such a manner that the oxygen is given up to a vacuum. Of these four, hæmoglobin has a wide distribution in vertebrate and invertebrate animals (Redfield, 1933), chlorocruorin is found only in certain polychaete worms (Fox, 1926, 1932, 1933), hæmerythrin in sipunculid worms (Florkin, 1933) and hæmocyanin in arthropods and molluscs (Redfield, 1934). The first three pigments named contain iron in the molecule, while the last has copper. Since the work of Peters (1912) it has been known that in oxyhæmoglobin one molecule of labile oxygen is united to one atom of iron. For oxyhæmocyanin Dhéré (1916, 1919) first showed the approximate proportionality between the oxygen and copper contents, and later Begemann (1924), Redfield, Coolidge, and Montgomery (1928) and Guillemet and Gosselin (1932) established the ratio of one molecule of labile oxygen to two atoms of copper. For oxyhæmerythrin it has recently been shown by Florkin (1933) that one molecule of oxygen is united to three atoms of iron. Up to the present, however, the ratio of oxygen to iron in oxychlorocruorin has not been known, and the work reported below was undertaken to settle this question. The quantity of iron present in the blood of *Spirographis spallanzanii*, both combined with chlorocruorin and free, has been analysed, and, the total oxygen capacity of the blood being known, the oxygen to iron ratio of oxychlorocruorin has been determined.

For iron analysis pure undiluted blood was extracted from the crown* of *Spirographis* at the Tamaris Marine Biological Station.† The technique already described (Roche and Fox, 1933) was used for obtaining the blood, with this difference, that the crown was amputated by means of a glass coverslip so that no iron instrument came into contact with the blood. Eight measured specimens (0·2 cc each) of blood, each derived from several individual worms, were diluted with distilled water and sealed in test tubes.

The iron estimations were made subsequently in Birmingham by the dipyridyl colorimetric method of Hill (1930). Hydrogen peroxide was first used to liberate the iron from the chlorocruorin and at the same time to render the solution colourless (Hill and Keilin, 1933). The method was found to be improved by heating the contents of the experimental test tubes in a water bath after adding the hydrogen peroxide and again after adding the hydrosulphite, and then waiting an hour before matching the colours with the standards.

Six iron estimations were made on each specimen of blood. The mean values for the iron contents of the eight blood specimens were as follows: 5·3, 5·1, 4·5, 4·6, 5·4, 4·6, 4·9, and $4 \cdot 9 \times 10^{-3}$ gram-atoms Fe per litre. The mean of these values is $4 \cdot 9 \times 10^{-3}$. The differences between the values for the separate blood specimens are doubtless due in part to experimental errors, but they may probably be caused in part also by differences in the chlorocruorin content of the bloods of individual worms. Much greater differences than would be required to account for the divergencies in the iron values found for *Spirographis* are known to occur in the hæmoglobin content of the blood of individuals of another polychaete, *Arenicola*, in which the total oxygen capacity, and therefore the hæmoglobin content, varies from 5·7 to 8·7 volumes per cent (Fox, 1926).

The iron present in the blood of *Spirographis* is partly combined in chlorocruorin and partly free. The next step in the investigation was to determine what proportion of the iron is free. This was done as follows. Sodium hydrosulphite devoid of iron impurity was added to known dilutions of *Spirographis* blood, with the result that the chlorocruorin was precipitated

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[*] The word "crown" is used here for the so-called gills, which morphologically are palps.

[†] My best thanks are due to Professor H. Cardot, Director of the Station, for his hospitality and help.

and at the same time the free iron was reduced to the ferrous state. After centrifuging to separate the precipitate, αα-dipyridyl crystals were added and the resulting pink solution was matched with solutions of ferrous iron of known concentrations containing dipyridyl. The mean value obtained in this way for the free iron in *Spirographis* blood was 5×10^{-5} gram-atoms per litre. The free iron is thus only 1% of the chlorocruorin iron, and this allows the total iron content of the blood given above to be taken as equal to the chlorocruorin iron.

Spirographis blood saturated with oxygen at atmospheric pressure contains 102 cc of oxygen per litre combined as oxychlorocruorin (Fox, 1926); that is, $4 \cdot 6 \times 10^{-3}$ gram-molecules O_2 per litre. The mean iron content of the chlorocruorin in the eight blood specimens analysed was $4 \cdot 9 \times 10^{-3}$ gram-atoms per litre. The ratio of the number of molecules of oxygen to atoms of iron in oxychlorocruorin is thus $4 \cdot 6/4 \cdot 9 = 0 \cdot 94$. Allowing for experimental error, this may be taken as unity, one molecule of labile oxygen corresponding to one atom of iron as in hæmoglobin.

The fact that the proportion of iron to oxygen was found to be higher in oxychlorocruorin than it should have been if the ratio were really unity may, however, not be due to experimental error but to the presence in the blood of a certain amount of metachlorocruorin or other derivative of the pigment. None such was detected spectroscopically in *Spirographis*, but the blood of some young specimens, 5 cm and less in length, of *Bispira voluticornis*, another large sabellid, is brown, not green. This brown colour is due to an admixture with the oxychlorocruorin of a pigment having a strong narrow absorption band at 561 mμ. Addition of sodium hydrosulphite does not alter this band. Addition of pyridine gives two α-bands of hæmochromogen at 582 and 557 mμ respectively. The first of these is derived from chlorocruorin, the second coincides with the pyridine-hæmochromogen of hæmoglobin. This presence of a hæmoglobin derivative in blood containing chlorocruorin is remarkable in itself. The hæmoglobin derivative is not visible in the blood of larger *Bispira*.

While the ratio of oxygen to iron is the same in oxychlorocruorin as in oxyhæmoglobin, chlorocruorin has a higher proportion of iron to other elements in its molecule than has hæmoglobin (Roche and Fox, 1933). Chlorocruorin may thus be regarded as the better transporter of oxygen. The lack of success of chlorocruorin in evolution, in spite of this superiority, may perhaps be due in part to its high molecular weight (Svedberg, 1933) preventing it from entering blood corpuscles.

2. *The Total Amount of Oxygen Carried by Chlorocruorin in* Spirographis

The total quantity of chlorocruorin in individuals of *Spirographis* was determined as follows. In each experiment undiluted blood was extracted from the crowns of several large individuals by the method previously described (Roche and Fox, 1933). Of this blood, exactly 0·097 cc was diluted to 8 cc with distilled water. To 2 cc of the diluted blood, 15 cc acetone containing 0·3 cc N HCl was added. After centrifuging, the volume of the resulting clear light brown solution (*a*) of chlorocruorohæmatin was measured.*

Next, a single worm was weighed, after which it was cut up with scissors, the fragments ground with sand, water added, and the suspension centrifuged. The volume of the liquid from the centrifuge tube, which contained the total blood of the worm, was measured. Then 2 cc of this liquid was added to 15 cc acetone containing 0·3 cc N HCl. As before, the resulting solution (*b*) of chlorocruorohæmatin was centrifuged and its final volume measured.

After the relative concentrations of hæmatin in the two acetone extracts (*a* and *b*) had been determined with a colorimeter, the necessary data were available for calculating the total quantity of chlorocruorin in the worm in terms of the quantity in 0·097 cc of blood. But 0·097 cc of blood contain $4 \cdot 7 \times 10^{-7}$ gram-atoms of chlorocruorin iron, since, as shown above, the blood contains $4 \cdot 9 \times 10^{-3}$ gram-atoms of iron per litre. Thus the total quantity of chlorocruorin iron in each worm could be directly deduced. The results of the investigation of 11 worms are given in Table I.

Table I—Chlorocruorin Content of the Blood of Individuals of *Spirographis*.

No. of worm	Wet weight† of worm, grams	Total chlorocruorin iron, gram-atoms $\times 10^{-6}$	Chlorocruorin iron per gram worm, gram-atoms $\times 10^{-7}$
1	2·1	0·6	2·9
2	3·2	0·9	2·8
3	3·8	1·6	4·2
4	4·1	1·3	3·2
5	4·9	1·5	3·1
6	5·1	1·7	3·3
7	5·6	1·2	2·1
8	5·6	1·5	2·7
9	6·4	1·5	2·4
10	8·0	3·2	4·0
11	8·1	2·1	2·6
		Mean	3·0

† In *Spirographis* the mean ratio of wet weight : dry weight : volume is 1 : 0·27 : 0·94.

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* It was first attempted to extract the hæmatin in alcohol and ether, but, probably owing to impurities in the ether, the colour of the extracts faded rapidly.

It is probable that the values for iron given in Table I are a little too high, for the following reason. Acetone added to the liquid obtained by centrifuging the suspension in water of the ground-up worm dissolves not only the chlorocruorohæmatin but also a certain amount of other pigments from the tissues of the worm. As a result, the acetone extract from the worm is of a slightly yellower brown than that from the whole blood. This must involve a small but indeterminable error in the colorimeter readings.

It will be noticed that the figures in Table I for the chlorocruorin iron per gram of worm vary considerably. In all probability this variation is not due to experimental error alone, but is in part real. In order to determine the experimental error of the method, a series of six separate acetone extractions was made from one and the same watery solution resulting from centrifuging the suspension in water of a single ground-up worm. These parallel samples gave values for the chlorocruorin content of the worm of which the highest was only 1·15 times the lowest. Yet the highest figure in the last column of Table I is twice the lowest figure. Such variations in the chlorocruorin content of the blood of different individuals would, as pointed out above, not be unexpected.

The quantity of oxygen which the total amount of chlorocruorin in the blood of an individual *Spirographis* could carry can now be deduced. As shown in Table I, the mean content of chlorocruorin iron per gram of worm (wet weight) is $3 \cdot 0 \times 10^{-7}$ gram-atoms. This means that a worm weighing 1 gram possesses in its blood a quantity of chlorocruorin which could bind $3 \cdot 0 \times 10^{-7}$ gram-molecules of oxygen. But the chlorocruorin in *Spirographis* blood in equilibrium with air at 17° C* is only 90% saturated with oxygen (Fox, 1926). Therefore the worm weighing 1 gram could carry $2 \cdot 7 \times 10^{-7}$ gram molecules, or 6·0 cmm, of oxygen attached to its chlorocruorin.

4. *Summary*

(1) One molecule of labile oxygen in oxychlorocruorin corresponds to one atom of iron. In this ratio of oxygen to metal, chlorocruorin resembles hæmoglobin and differs from hæmocyanin and hæmerythrin.

(2) The total chlorocruorin content of *Spirographis* blood and the quantity of oxygen which the blood can carry have been determined.

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* By an oversight this temperature was omitted from my paper of 1926.

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The Decomposition of Sodium Formate by Bacterium coli *in the presence of Heavy Water*

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Since the discovery of the heavy hydrogen isotope, it was realized that this might prove very useful for the investigation of the mechanism of chemical and biological reactions.

As a simple reaction of a biological nature, we have chosen the decomposition of sodium formate by the enzyme hydrogenlyase of *Bacterium coli* (see Stephenson and Stickland, 1932).

The mechanism of the decomposition of the formate generally accepted has been

$$HCOONa + H_2O \rightleftharpoons HCOOH + NaOH \qquad (1)$$

$$HCOOH \rightarrow H_2 + CO_2 \qquad (2)$$

Since both formate and water are involved in this reaction, it was thought that some idea of the mode of decomposition of the formate could be gained by a study of the isotopic composition of the evolved hydrogen in the presence of varying amounts of heavy water.

If the reactions (1) and (2) were the only ones involved then, by replacing normal water by heavy water, the reactions

$$HCOO\,Na + D_2O \rightleftharpoons HCOOD + NaOD \tag{1A}$$

$$HCOOD \rightleftharpoons HD + CO_2 \tag{2A}$$

would result, *i.e.*, only the acid — H — atom of the formate could be exchanged for D.

Thus the D-content of the evolved gas can never exceed half that of the water, also D_2 molecules should not be evolved.

To examine whether exchange between the carbon linked H of the formate and the D of the heavy water takes place, the following experiment was made: 0·6 gm HCOONa and 0·05 gm of water containing 37% D were mixed at room temperature, and after five hours the water was distilled off from the salt and its D-content re-determined. It was found that no appreciable change had taken place, thus the H atom of the HCOO ion is not replaced by D by dissolving formate in heavy water. This result is in agreement with the experiments of Bonhœffer and Brown (1933) and Bonhœffer and Klar (1934), which showed that the carbon-linked hydrogen atoms of organic compounds are not replaced by heavy hydrogen in solution, provided catalysts are absent.

The analysis was carried out by the methods described in a previous communication (Farkas, 1934), using a micro thermo-conductivity method for the determination of the D-content of the hydrogen gas. If water was to be analysed, it was decomposed on a hot tungsten wire into hydrogen and tungstic oxide, and the D-content of the resulting gas analysed by the micro method.

To gain some information about the mechanism of the mode of decomposition of the formate, three sets of experiments were carried out :—

(1) The enzymatic decomposition of sodium formate in the presence of different amounts of heavy water.

(2) The catalysis of the reaction $H_2O + HD \rightleftharpoons HOD + H_2$ by the enzymes present in suspensions of *Bact. coli*.

(3) The decomposition of formic acid by palladium black in the presence of heavy water, to compare the action of the enzyme with that of palladium.

For the experiments (1) and (2) the *Bact. coli* was grown on a tryptic digest of caseinogen containing 0·5% formate in a flask, and from this a washed suspension was made (see Yudkin, 1932). The bacteria were dried as thoroughly as possible by pouring off the water after centrifugation, in order to avoid excessive dilution of the heavy water, when the bacteria were put in the sodium formate heavy water solution.

The enzymatic decomposition of the sodium formate in heavy water was carried out in the following way: 0·1 cc M/20 phosphate buffer of p_H 7 and 0·1 cc M/10 sodium formate were placed in a tube, and dried by evaporation. Then about 0·3 cc of water of known D-content and about 0·05 cc of the nearly dry bacterial suspension were added. The tube was cooled to 0° C and evacuated. It was then warmed to 38–40° C for some hours, during which time 0·1–0·2 cc of gas at (N.T.P.) were evolved, exerting a few millimetres pressure in the tube.

The D-content of the evolved hydrogen was measured several times, and finally the D-content of the water was determined in order to estimate the dilution of the original water caused by the addition of the bacterial suspension.

In order to test the method in the first experiment, sodium formate was decomposed by the bacteria in presence of normal water. After the carbon dioxide and water had been frozen out by means of liquid air, the evolved gas was found to consist of pure hydrogen and contained no uncondensible impurities. Table I shows the results obtained in heavy water of different D-content.

Table I

D content of the water in %	D content of the gas in %	$\frac{(H/D)\ \text{gas}}{(H/D)\ \text{water}}$	K_3
0	0	—	–
19·5	6·5	3·5	3·71
25·0	10·0	3·0	3·06
30·7	12·5	3·1	3·19
77·5	49·1	3·5	4·00
	Mean	3·3	3·50

The expression given in the third column is the ratio of the relative concentrations of hydrogen and diplogen in the gas and in the water, *i.e.*, for the first experiment:—

$$\frac{93\cdot5}{6\cdot5} \div \frac{80\cdot5}{19\cdot5} = 3\cdot5.$$

This magnitude is characteristic for the isotopic composition of the gas, being nearly independent of the concentration of H and D in the water, and is, as we shall have occasion to observe, approximately the equilibrium constant of the reaction :—

$$H_2O_{(liq.)} + HD \rightleftharpoons HOD_{(liq.)} + H_2 \qquad (3)^*$$

A more detailed analysis of the hydrogen evolved with regard to its composition in respect to the three different molecular species, H_2, HD and D_2 (*loc. cit.*), showed that these three constituents were always present in their equilibrium concentration, *i.e.*, according to the equation

$$K_1 = \frac{[HD]^2}{[H_2][D_2]}, \qquad (4)$$

K_1 being the equilibrium constant equal to 3·28 at 40° C (see Urey and Rittenberg, 1933).

Thus the reaction schemes indicated by equations (1A) and (2A) must be rejected since neither of the anticipated results are fulfilled.

To investigate the effect of *Bact. coli* on the pure exchange reaction of equation (3), the evolved hydrogen diplogen mixture (*e.g.*, containing 12 % D) was left in contact with the water from which it had been liberated (*e.g.*, containing 30·7 % D) in the presence of bacteria. In this experiment, even after a day, no change whatever in the isotopic composition of the gas occurred. In another series of experiments, normal water was left in contact with hydrogen containing about 30% D in presence of bacteria (without addition of formate and buffer solution), when in a few hours the hydrogen in the gas was completely† replaced by ordinary hydrogen, for certain concentrations of heavy water give a gas with more than half the amount of D present in the water, while D_2 molecules are also found, according to the reaction.‡ In the third series of experiments, the bacteria were replaced by palladium black, and it was found that the decomposition of the formate and the reaction (3) took place in exactly the same way as with bacteria. If, *e.g.*, 0·3 cc water, containing 35 % D and 1 % formic acid, was decomposed by palladium black at 40° C, the hydrogen evolved contained 12 % D and the composition of the gas did not change when left in contact with the water in the presence of palladium black. On the other hand, starting with water containing 27 % D and ordinary hydrogen, the D content of the gas now gradually reached 10%, owing to the catalysis of the reaction (3) by palladium black (see Horiuti and Polanyi, 1933).

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* See Table I, column 4.

† This replacement of heavy hydrogen was complete, as the amount of heavy hydrogen present in the gas was negligibly small compared with the amount of ordinary hydrogen present in the water.

‡ These reactions do not occur in the absence of bacteria.

Discussion

The second series of experiments, *i.e.*, the catalysis of the hydrogen-water exchange reaction by the enzyme indicates definitely that the hydrogen evolved by the decomposition of formate, with regard to its D-content, is in equilibrium with the water from which it was liberated. This is obvious from the fact that the D-content of the evolved hydrogen did not change in contact with the water in presence of *Bact. coli*, although hydrogen containing about 27% D was readily transformed into ordinary hydrogen when in contact with ordinary water, in the presence of *Bact. coli*. Exactly the same observations were made for the decomposition of the formate by palladium—for example, hydrogen containing 12% D is in equilibrium with water containing 35% D.

The conditions of equilibrium between the different hydrogens and the different kinds of water are determined by the following equations :—

$$H_2 + D_2 \rightleftharpoons 2\,HD \quad K_1 = \frac{[HD]^2}{[H_2]\,[D_2]} \tag{4}$$

$$H_2O + D_2O \rightleftharpoons 2\,HOD \quad K_2 = \frac{[HOD]^2}{[H_2O]\,[D_2O]} \tag{5}$$

$$H_2O + HD \rightleftharpoons HOD + H_2 \quad K_3 = \frac{[HOD]\,[H_2]}{[H_2O]\,[HD]} \qquad (3)^*$$

K_1 was calculated theoretically by Urey and Rittenberg (1933) to be 3·28 at 20° C, which value was confirmed by the experiments of A. and L. Farkas (1933, 1934), and of Rittenberg, Bleakney and Urey (1934).

K_2 has not yet been determined, but to a first approximation it will not differ much from K_1.

K_3 was determined by Bonhoeffer und Rummel (1934), and found to be about 3.

Putting $K_2 = 4$ and introducing the H and D content of the gas—

$$H_{gas} = [H_2] + \tfrac{1}{2}\,[HD] \text{ and } D_{gas} = [D_2] + \tfrac{1}{2}\,[HD]$$

and the corresponding equations for water, we obtain the values for K_3 given

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[*] The equilibria in all other exchange reactions between water and hydrogen, *e.g.*, $H_2O + D_2 \rightleftharpoons D_2O + H_2$, can be derived from those three equilibria. The exchange reaction (3) proceeds according to Horiuti and Polanyi (1934) by the ionization of the hydrogen atoms formed on the surface of the catalyst.

in Table I, column 4. It will be seen that these do not differ sensibly from the ratio $(H/D)_{gas}/(H/D)$ water, and are in good agreement with the value obtained by Bonhœffer and Rummel. The experiment on the hydrogen-water exchange with palladium black gives $K_3 = 3 \cdot 58$.

With regard to the mechanism of the decomposition of formate by *Bact. coli*, one can state that the process involves atomic reactions, *i.e.*, reactions between atoms or radicals formed by the action of the enzyme on the formate and water, since the equilibria (3), (4), and (5) cannot be attained by reactions between the molecules H_2, D_2, HD, H_2O, D_2O, HOD (Rutherford and others, 1934 ; A. and L. Farkas, 1933).

It is difficult to formulate the special reactions involved in the decomposition, but to illustrate the state of affairs we might choose one example from a number of different possibilities :—

$$HCOO^- + HOD \xrightarrow{\text{enzyme}} HCOO + D + HO^- \qquad (7)$$

$$HCOO \rightarrow H + CO_2 \qquad (8)$$

That H and D-atoms are actually involved in the decomposition of formate by palladium black is fairly certain, since it is known that on the surface of palladium the hydrogen is really present in atomic form. It is very likely that the decomposition of formate in both reactions involves the same intermediate products, although the enzyme might induce the first step—production of the radicals and atoms in a way different from palladium black.

It must be emphasized that the difference in the D-content of the hydrogen evolved, compared with the D-content of the water, is not due to a difference in velocities of reactions involving light and heavy water, but to the position of the equilibrium in equation (3), and this equilibrium plays an important role so far not considered in the preferential liberation of light hydrogen in the well-known electrolytic and chemical methods of separation.

Summary

The isotopic composition of the hydrogen evolved from mixtures of heavy and ordinary waters with sodium formate by the action of the hydrogenlyase in *Bacterium coli* has been analysed. It is found that the composition of the liberated hydrogen is determined by the equilibria $H_2 + D_2 = 2\,HD$ and $H_2O + HD \rightleftharpoons HOD + H_2$. We must therefore conclude that the decomposi-

tion of the formate by the enzyme hydrogenlyase of *Bact. coli* occurs through atomic reactions, that the establishment of these equilibria between molecular hydrogen and water is catalysed by the *B. coli*, and that the primary formed H and D atoms exchange with the H and D atoms of the water before combining to form hydrogen molecules.

It is shown that palladium black behaves in a similar manner, and the importance of this equilibrium in the preferential liberation of light hydrogen in electrolytic and chemical reactions is emphasized.

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Unco-ordinated Contractions caused by Egg White and by alterations in the Cation ratio of the Medium in the Heart of the Chick embryo in vitro

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Introduction

In May, 1932, some experiments were made in which fragments of chick embryos in primitive streak stages were explanted into crude white of egg as culture medium. The object was to study hæmatopoiesis, which occurred in these explants (Murray, 1933). Only two of the cultures interest us here. Both were derived from embryos having pear-shaped *areæ pellucidæ* with primitive streaks but no head processes and each consisted of that part of the *area pellucida* of one side which lies opposite the posterior half or three-quarters of the primitive streak. Both cultures survived in the egg white and in each there was discovered, after two and five days incubation respectively, an area which contained actively contracting cells. The contractions were of small amplitude and there was no co-ordination between the cells. This activity persisted in one culture for 36 days.

It was necessary to have some name by which this anarchic contractility could be designated ; it resembled fibrillation, at least superficially, but it was thought best to avoid this term as the present phenomenon might prove entirely unconnected with fibrillation. The word "twitter," used as noun and verb, described the appearance rather well, and I have adopted it as a provisional name for this kind of activity.

The contracting cells undoubtedly represented the cardiac *anlagen*, which had undergone a degree of differentiation *in vitro* ; the site of origin of the explants was such as might cause one to expect the occasional inclusion of the *anlage* of the heart, and similar explants, cultivated in plasma and embryo extract, frequently show differentiation of contracting areas, but in such explants the contractions are of quite a different character, being fully co-ordinated pulsations. It was evident that egg white had in some way changed the character of the beat of heart cells so that the normal co-ordination was absent and the contractions were anarchic.

No further investigation was made for twelve months. The cause of this effect of egg white was then investigated and the results obtained form the subject of this paper.

METHODS

The technical methods used were essentially those of ordinary tissue culture. The explants were always cultivated as hanging drop preparations on cover-glasses. Except where the contrary is stated, embryo extract was not used in the media. Its function in ordinary tissue culture is to encourage growth, and in the present experiments growth was not desired but was rather to be avoided, as bringing with it dedifferentiation and loss of muscular contractility. Traces of embryo extract were, nevertheless, present in all media containing plasma, some small implement being dipped in the extract and then used to stir the plasma, to make it clot.

The principal difference from the ordinary methods of tissue culture was that the media were never changed, except in experiment 7. There were two reasons for this: firstly, it was unnecessary, as both the co-ordinate beat and twittering could be maintained for long periods (up to six weeks) without any change of medium, and secondly changing the medium tends to promote growth, which was to be avoided for the reasons given.

Table I—Solutions used—grams per cent

Designation	NaCl	KCl	$CaCl_2 6H_2O$	$MgCl_2 6H_2O$	Osmotic pressure calculated as equivalent to % NaCl	Electrometric p_H
Ordinary Pannett and Comptons' solution ..	0·48	0·06	0·03	0·05	0·56	7·63
PC (0·76) . . .	0·68	0·06	0·03	0·05	0·76	—
Sal EW ..	0·3	0·252	0·03	0·074	0·54	7·96
PC NaCl 0·3% .	0·3	0·06	0·03	0·05	0·38	—
PC (0·352) ..	0·3	0·04	0·02	0·03	0·352	—
PC (0·586) .	0·525	0·046	0·023	0·039	0·586	—

Table I shows the composition of the principal saline media used. All salines contained the phosphate buffer used in Pannett and Comptons' solution (1924). The determinations of p_H were electrometric, made with a quinhydrone electrode. All osmotic pressures are given, in table and text, in terms of osmotically equivalent solutions of NaCl; experimental determinations were not made, the figures given being calculated; the calculations assume complete dissociation of the salts and neglect the osmotic pressure of the phosphate buffer. The slight dilution of the solutions by the water of crystallization in $CaCl_2, 6H_2O$ and $MgCl_2, 6H_2O$ is also neglected.

PART 1—THE DIFFERENTIATION OF CARDIAC ANLAGEN IN EGG WHITE

The embryos from which explants were taken were, in this section, always in primitive streak to early head fold stages. The material explanted was not in all respects similar in all experiments, but always contained the cardiac anlagen. The differences were in size of explant ; sometimes the entire *area pellucida* was explanted, at others only the anterior half or large parts of this. It was found that if the explants were too small they were unable to survive in the egg white, but if they were large enough to form vesicles survival usually occurred. Explants below the minimal viable size are excluded from the present account.

The egg white used as medium was always the more fluid white of the egg ; the more viscous white being more difficult to handle. When egg white was taken from incubated eggs, fertile eggs were used.

Experiments 1–5—In these experiments the media were : egg white from fresh eggs (14 explants), from eggs incubated overnight (21 explants), from eggs incubated for two days (9 explants), from four-day eggs (13 explants), and from five-day eggs (8 explants). In addition, thirteen explants were made in plasma and embryo extract to serve as controls.

All the control explants survived and in four there appeared an area of cells pulsating rhythmically and in co-ordination. In egg white from fresh eggs, or from eggs incubated for less than four days, the explants showed themselves able to survive and, at least in overnight and two-day egg white, to undergo a degree of early differentiation, forming brain, spinal cord, somites, blood, etc., but no contracting cells appeared. But in three explants in egg white from four-day eggs, and in six in egg white from five-day eggs there appeared areas of twittering cells. Co-ordinate beating never appeared in egg white.

The following was the course of events in those explants in which twittering areas appeared : after 24 hours the explants had become vesicular, the walls of the vesicle being in appearance epithelial. The vesicles contained fluid, and generally opaque masses could be seen inside, while, especially when the entire *area pellucida* was explanted, differentiation might occur, halting at about the stage of a two-day embryo. When contracting tissue appeared it seemed to form part of, or else to lie just below, the wall of the vesicle. The amplitude of movement was very small and the contracting area was usually, especially at first, very restricted, so that careful search was necessary for its discovery.

Twittering was usually discovered at two days after explantation; it may nevertheless have been present before this as the cultures were left undisturbed on the day after explantation in order to avoid detachment of the still loosely attached explants from the cover-slip. It often commenced later, and once not until 12 days after explantation. The length of time during which contractile activity was maintained varied considerably, ranging from only one day in four cultures to 18 days in one and 12 in another.

In the longer surviving explants of the two in the original observation, survival and continuation of twittering was very long, 36 days, and the culture spread as a whole over the cover-slip so that the contracting cells could easily be studied. In the later experiments such complete spreading never occurred, the great bulk of each explant remaining intact in the centre, being surrounded only by a very thin zone of migration composed mainly of flat epithelial cells. The twittering tissue always remained in the central, organized zone, where it could be seen only with some difficulty and where it was quite unsuitably placed for detailed study.

For the proposed investigation of the cause of twittering some other material was required, which would provide greater quantities of twittering tissue and which would arrange itself in a manner more suitable for the study of the active cells. Such material was found in the entire hearts of two to three day embryos, having from 20 to 40 somites. At this period the heart has already been beating for some time, and has the form of a bent tube, the bend being in the ventricle, and the convex side being the future apical side of the ventricle. The atria are present as two pouches which are relatively larger in the older than in the younger embryos. The atrio-ventricular canal is a constriction between the atria and the ventricle, while the conus is a comparatively narrow tube continuing the arterial end of the ventricle. The ventricular wall is little thickened in the younger embryos but in the later part of the period its convex side has undergone considerable muscular elaboration. Needham's "Chemical Embryology," fig. 90, p. 545, contains excellent illustrations of stages in the development of the heart; the stages used in the present work range from that represented by the figure 2·6 to that shown by the figure 3·2.

PART 2—THE EFFECT OF EGG WHITE ON THE ENTIRE HEARTS OF 2-3 DAY EMBRYOS

In all the experiments of this section the technique was as follows: the medium was the more fluid part of the egg white from fertile eggs incubated for the desired period. The hearts to be explanted were dissected from the embryos in NaCl 0·75% or in Pannett and Comptons' saline solution, and explanted into the egg white as a hanging drop preparation in the usual manner.

Experiment 6—The medium was egg white from a fertile egg incubated for two days: eight explants were made. The co-ordinate beat stopped immediately in all the explants, and it may here be finally stated, that the co-ordinate beat always stopped at once when the medium was pure egg white.

Most of the explants became distinctly unhealthy with many cells rounded up and dead; it is probable that some explants died completely. All explants remained motionless, with one exception, in which twittering occurred and continued for 18 days. It is probable that the entire heart was never active. It is not known why this one heart should have behaved in so satisfactory a manner while all the other seven remained motionless.

Experiment 7—The medium was egg white from fertile eggs incubated for four days. Twenty-three explants were made. In a small number of explants the medium was, at six days after explantation, removed and replaced with fresh; this seemed to have no particular effect, either good or bad, and in all other experiments the medium has been left unchanged.

Twitter was observed in 13 explants, but the number in which it actually occurred was probably larger as, owing to illness, the behaviour of the explants could not be as thoroughly studied as was desired. This figure should therefore be regarded as a minimum. One explant twittered for each of the periods 29, 22, 19 days, two for 14 days, three for 11 days, two for 9 days, and the remainder for shorter periods.

In this experiment, and also in the single instance of twittering observed in experiment 6, twittering frequently occurred with great activity while the explants spread into relatively thin sheets over the cover-glass. In such cases there was always a thicker central region in which movement was absent or not very active and this was surrounded by a zone of migration, composed in its inner region of several layers of cells forming a network and more peripherally of a very flat sheet of cells lining the cover-glass. The inner, thicker part of the zone of migration was the seat of most active movement, the flattened cells of the peripheral zone being usually stationary, though in the more active explants even some of these were found, by careful examination, to be making contractions of small amplitude.

Experiment 8.—The medium was egg white from a fertile egg incubated for five days. Fifteen explants were made. All explants were examined on the day of explantation at about one to three or four hours after the operation, again on each of the two following days, and on succeeding days somewhat less frequently. Twittering was seen on the day of explantation in all cultures except three, in eleven on the second day and in seven on the third day. Twittering continued in one culture for 35 days, in two for 31 days, in three for 20 days, in one for 6 days, in three and probably four for 2 days, and in four for only 1 day.

On the day of explantation, twittering was in four cultures generally distributed and not confined to any particular region of the heart; in three the heart was stationary. In the remaining eight there was no movement in the conus but varying amounts of twittering in the sino-atrial and ventricular regions. On the second day there was twittering in 11 hearts and in 7 of these the activity was generally distributed over the heart. In one other heart twittering was nearly general, but the convex (apical) side of the ventricle was stationary. Of the remaining three, one was twittering in the sino-atrial region and part of the ventricle, one in the sino-atrial region, and one had slight movements in the conus. The four remaining hearts were stationary. On both the third and fourth days seven explants were twittering, and in all the twitter was generally distributed over the heart.

From these facts it may be seen that egg white can cause twittering in all parts of the heart, but from observations made on the first and second days, that it does so most readily in the sino-atrial and ventricular regions, less readily in the conus.

The frequency of contraction in twittering was always greatest in the sino-atrial region and least in the conus.

Variation in the Effect of Egg White according to Age

The experiments with hearts from 2–3 day embryos agree with those in which fragments of younger embryos were used, in indicating that the egg white from five-day eggs produces twittering more readily than that from fresh eggs or from eggs incubated for only two days instead of five.

PART 3—THE CAUSE OF TWITTERING IN EGG WHITE

Twittering in egg white might be caused by p_H, by the rather low osmotic pressure of egg white, by some chemical factor present in egg white, or by the absence from egg white of some substance necessary for the maintenance of the normal beat.

1—p_H

The p_H of the ordinary tissue culture medium (plasma and embryo extract) is about 7·5; in such a medium co-ordinate beat is maintained and there is no twittering. The available measurements of the p_H of egg white do not indicate any very great difference from this value. There is general agreement among the authors that the p_H of egg white falls during development, being, according to Aggazzotti a little over 7·5 at five days, according to Vladimirov about one unit higher, while following Buytendijk and Woerdemann that of the less viscous component (falling faster than that of the more viscous) would be 8·0, or perhaps a little over. These facts are in themselves sufficient to exclude p_H as a cause of twittering in egg white (data from Needham).

2—*Osmotic Pressure*

Bialascewicz, Rice and Young (both references from Needham), Bateman (1932), and Hale (1933) have obtained values of the depression of the freezing point of egg white ranging from about —0·450° C at four days, and —0·440° C at six days for the first author to between —0·42° C and 0·41° C for the last (unincubated eggs). This last figure is equivalent to a concentration of NaCl of about 0·71%. During incubation osmotic pressure rises; hence it is very improbable that, at four or five days, it is as low as 0·71% NaCl. In spite of this, however, the osmotic pressure of pure egg white is considerably below that of a plasma and embryo extract clot. I have accepted equivalence with 0·71% NaCl at the osmotic pressure of egg white at four or five days because it can safely be regarded as a minimum. If normal media having an osmotic pressure at this level maintain the co-ordinate beat and do not cause twittering, hypotonicity can be excluded from the possible causes of twittering in egg white.

It may be noted that the tonicity of 0·71% NaCl is little below that of 0·75% NaCl, which is normal saline and does not cause twittering.

Experiment 9—Seven hearts of two-and-a-half day embryos were explanted into a medium which consisted of nine parts of the saline PC (0·586) mixed with one part of plasma. Clotting was induced by stirring with a rod dipped in embryo extract. The calculated osmotic pressure of the mixture, accepting Bialascewicz's figure for the depression of the freezing point of adult fowl blood (—0·635° C), is approximately equivalent to that of 0·717% NaCl, or very slightly above that of fresh egg white and probably below, or at most equal to, that of four or five day egg white. Co-ordinate beating occurred in all the explants and twittering did not occur.

The following facts also have a bearing on the question.

(1) As will be described under experiment 12, active twittering was induced and co-ordinate beating prevented in hearts explanted into a medium which consisted of the exudate from a clot composed of nine parts of a solution called Sal EW KCl 0·3% and one part of plasma. In a medium similar in all respects save that the saline component contained KCl 0·2%, instead of 0·3%, there was very much less twittering and a co-ordinate beat occurred in several hearts. Thus here twittering varied directly, and beating inversely, with the osmotic pressure, exactly the opposite of the result expected if hypotonicity is the cause of twittering in egg white. A similar conclusion followed from the results of experiment 14.

(2) In another experiment the two media used consisted of egg white diluted with ordinary Pannett and Comptons' solution in ratios of one to four and four to one. The osmotic pressure of this saline is approximately equivalent to that of 0·56% NaCl; thus egg white diluted with four times its volume of the saline is hypotonic to pure egg white and to egg white diluted with one-quarter of its volume. Nevertheless, there was more twittering in the higher osmotic pressure than in the lower, and co-ordinate beating occurred in the lower and not in the higher.

(3) Further, in experiments to be described in another paper, the media consisted of the saline Sal EW, modified by having its content of KCl raised in one experiment to 1·2% and in another to 1·8%, mixed with plasma in the ratio of five to one. The osmotic pressures of the solutions were calculated as equal to those of NaCl 1·28% and 1·75%, so that the mixtures with plasma were very hypertonic to egg white. Nevertheless, very active twittering occurred in both.

From these facts it is concluded that the rather low osmotic pressure of egg white is neither the cause, nor a necessary condition of twittering. This conclusion does not imply that osmotic pressures lower than that of egg white cannot cause twittering; experiments 15 and 16, indeed, show that strongly hypertonic media can, as a sort of shock effect, cause the appearance of a transient twitter.

3—*The Action of Inorganic Cations*

The exclusion of p_H and osmotic pressure from the list of possible causes of twittering in egg white left the two possibilities of absence from egg white of some substance necessary for the maintenance of the co-ordinate beat and of the presence in egg white of an agent responsible for its production. In subsidiary experiments, which need not be described, evidence was found which favoured the second possibility rather than the first.

An obviously possible twitter-producing agent would be some unusual balance of inorganic ions, and the available estimates of the ash of egg white showed that the free cations were probably present in concentrations very different from those in a balanced solution of salts.

According to Iljin (from Needham), the ash of egg white contains the cations Na, K, Ca, Mg in the following proportions (milligrams per hundred grams wet weight):—

Na	K	Ca	Mg
123·8	132·6	9·8	8·5

When this ratio of cations is compared with that of a balanced saline solution, it is seen that there is little sodium but relatively large quantities of potassium. With a view to investigating the effects of so remarkable a cation ratio on the behaviour of hearts *in vitro* a solution was prepared which would have the cations present in the same concentration. This solution, which I call Sal EW had the following constitution (grams per cent.):—

NaCl	KCl	$MgCl_2\,6H_2O$	$CaCl_2\,6H_2O$
0·3	0·252	0·074	0·03*

To this there was always added the phosphate buffer used in ordinary Pannett and Comptons' solution. The p_H was found electrometrically to be 7·96 and the osmotic pressure was about equal to that of 0·54% NaCl. The p_H of ordinary Pannett and Comptons' solution was found to be 7·63; its osmotic pressure was calculated as equivalent to that of 0·56% NaCl.

The most obvious manner of testing the effect of this solution was to use it as a sole medium or after the addition of glucose. A number of experiments were performed in this manner but the method was abandoned when it was found that many of the most interesting effects of this and related solutions were obtained several days after explantation; explants in pure saline or saline-glucose solutions do not survive for this length of time.

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* An error was made in regard to $CaCl_2\,6H_2O$; the amount ought to have been 0·06; an experiment with this concentration was performed (experiment 14).

Experiment 10—In the first experiment with Sal EW two media were used. Medium A consisted of one part of the white of a fertile egg incubated for four days mixed with four parts of Sal EW; medium B was similarly constituted except that the diluting saline was PC (0·76). Eight hearts were explanted into each medium. The explants were examined daily until day 9, thereafter on days 11 and 14.

Results—(1) Twitter always occurred in medium A. It always appeared first in the sino-atrial region, and as time went on tended to spread into the ventricle, especially along its convex side (prospective apical side), so that it tended to become generally distributed through the sino-atrial and ventricular regions; it never became general throughout the heart because the conus never showed more than a few twittering cells and frequently had a co-ordinate beat. It persisted for periods ranging from 5 to 11 days.

A true co-ordinate beat never occurred in the sino-atrial and ventricular regions, but a curious condition of partial co-ordination of the twitter appeared in four and perhaps one other culture, but not in the remaining three. It is not proposed here to give a full description of this phenomenon or to discuss its significance; but it may be said that it appeared to be a subjection of the short amplitude anarchic twitter contractions to the influence of a co-ordinating agent, such as an electric impulse, and not to be a conversion of the twitter into a true co-ordinate beat of long amplitude contractions. In three of the four cultures the co-ordination was imposed upon previously unco-ordinated twitters and in all the co-ordination disappeared again before the cessation of twittering; it always appeared first in the higher parts of the heart and might spread over the whole sino-atrial and ventricular regions. Co-ordination was never perfect and the contractions always resembled those of twitter rather than those of the normal beat.

In medium A there occurred, at some time during the life of the cultures, a co-ordinate beat confined to the conus. This was always a slow beat, as is the beat of this region when isolated from the rest of the heart in a normal medium. It never occurred on the first day, on which all cultures in this medium were stationary. On the second and third days it was present in seven cultures, on the fourth day in only two, and thereafter it persisted in two (with some interruption in one) until the eighth and ninth (doubtfully eleventh) days.

(2) In medium B, twittering occurred in only one culture, on only one day, and was confined to a very small region of the sinus. Co-ordinate beating occurred in all cultures on the first, second and succeeding days, except in one which was damaged on the second day. There was perhaps a tendency for beating to be stronger in the conus than in other parts of the heart, and in two hearts the beat was on the first day confined to the conus. The frequency of beating was often irregular and the degree of irregularity might vary in the same heart from time to time.

Conclusions—(1) The differences in behaviour between the two groups, and in particular the occurrence of twitter in medium A, were due to the different concentrations of cations in the two media.

(2) Egg white contains nutritive materials sufficient for the maintenance of the co-ordinate beat for some days; subsidiary experiments showed that in a pure balanced saline solution the beat cannot be maintained for much longer than 24 hours. The cessation of co-ordinate beating in pure egg white is therefore not due to nutritional deficiency.

(3) The co-ordinate beat was more resistant to the inhibitory action in the conus than elsewhere.

(4) Twittering was more readily produced in the sino-atrial region than elsewhere, and least readily in the conus.

(5) If the only agent in egg white which is concerned in the production of twitter is one or more of the free ions sodium, potassium, magnesium, calcium, dilution of egg white with a saline solution whose composition in respect of these ions was identical with that of egg white, should affect the heart exactly as does egg white. Egg white diluted with Sal EW, however, does not exactly reproduce the effects of pure egg white, for it tolerates a co-ordinate beat in the conus and some degree of co-ordination of the twittering cells in other parts of the heart. There is, of course, no reason for supposing that Sal EW does exactly reproduce the free cation constitution of egg white, for it is based upon the constitution of the ash, and, further, upon that of the ash of fresh eggs and not of eggs incubated for four or five days, and it is with the latter egg white that the comparison should properly be made. In spite of this discrepancy, a remarkably close reproduction was achieved in regard to the production of twitter, for not only was twittering induced, but it was induced in those regions of the heart in which it is most commonly produced by egg white.

Experiment 11—The conclusions from experiment 10 were in one respect open to criticism; it might be argued that the cause of twittering in egg white was not the curious ratio of inorganic cations, but some other factor whose action was prevented in the presence of a properly balanced solution of salts. This criticism would be answered only when it was shown that twittering could be produced, in a medium from which egg white was itself absent, by a solution containing cations in the necessary ratio. The present experiment was the first, incompletely successful, attempt to carry out this test.

Two media were used. Medium A consisted of one part of plasma to five parts of Sal EW, medium B of one part of plasma to five of PC (0·76). The media were clotted by stirring with a rod which had been dipped in embryo extract. Ten hearts were explanted into medium A, four into medium B. The explants were examined on the following days: 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 15. Many explants were, on the fifteenth day, still not stationary, but it was unfortunately impossible to continue observations beyond that day.

Results—(1) When examined immediately after explantation, all explants in medium A showed twittering and none were beating, while all in medium B were beating and none were twittering. At the second examination on the same day, after incubation, all explants of both groups were beating, none twittering. Beating continued for a number of days in both media.

(2) Many irregularities occurred in the co-ordinate beat in medium A; perhaps the commonest were heart-block, irregular frequency in the sinus, disco-ordination between centres of beating in the sinus and atria, and a peculiar condition which I called "twitteroid." Heart-block might occur between atria and ventricle, or between sinus and atria; all degrees of block occurred from 2 to 1 to much higher orders, and it was frequently very irregular, varying in severity in the same heart from time to time. Disco-ordination between centres of beating in the sino-atrial region appeared as a failure of a centre of beating in the sinus, and of a similar centre in each of the two atria, to beat together, resulting in extremely chaotic contractions of the whole sino-atrial region. In addition to this, the sinus itself often displayed irregularity in the frequency of contraction.

"Twitteroid" was a condition, nearly always confined to the sinus, of imperfectly co-ordinated and very chaotic beating, in which there appeared to be two elements involved: (*a*) contractions of the normal type, of long amplitude, imperfectly co-ordinated, and (*b*) unco-ordinated small amplitude contractions of twitter type, which to some extent interfered with the long amplitude contractions, increasing their irregularity and further reducing their co-ordination. Twitteroid, which occurred at some time in every explant in medium A, always followed more normal beating and was in five explants succeeded by twittering.

Twittering, apart from that immediately following explantation, occurred in six hearts in medium A, always late in the history of the explant and in five succeeding twitteroid when the co-ordinate element in the latter had disappeared. If the interpretation of twitteroid as a mixture of twittering and beating is correct, twittering occurred in every explant in medium A, at first disguised by association with a co-ordinate beat, as twitteroid, and in five explants it became obvious later with the disappearance of the co-ordinate element. Twittering was usually, if not always, confined to the sino-atrial region, of which in some cultures it involved the greater part, in others only a few cells. In one explant it commenced comparatively early (sixth day) in a thin membrane of small flat cells which formed part of the zone of migration and was free from the influence of the co-ordinate element in the twitteroid sino-atrial region.

(3) The control explants, in medium B, showed far less interference with the regularity of beating than those in medium A, and disco-ordination in the sino-atrial region, twitteroid and twitter did not occur. On the other hand, intermittency of beating was perhaps more common in medium B than in A.

Conclusions—The solution Sal EW caused twittering in mixture with plasma and in the absence of egg white; it is concluded that twittering in egg white is due to its cation balance. The solution also in other ways disturbed the co-ordination of the beat.

Experiment 12—The constitution of the solution Sal EW suggested that the high content of potassium was probably important as a cause of twittering. The present experiment was designed to test this hypothesis. A somewhat different technique was used from that of the last experiment. Three saline solutions were prepared: one was Sal EW, and the other two differed from this only in containing 0·2% and 0·3% KCl respectively. The media were prepared thus: nine parts of each saline were mixed each with one part of plasma and the mixture was clotted by stirring with a rod which had been dipped into embryo extract; the clots were then cut into several fragments with a knife and incubated for about 10 minutes. The fluid exudates which then appeared were used as media. The exudate containing 0·3% KCl was called medium A, that containing 0·25% KCl medium B, that containing 0·2% KCl medium C. The technique of explantation was that usual for explants in fluid media. These media have, of course, a lower nutrient value than those in which whole plasma is included. Further, tissue cultures in general seem to thrive less well in a fluid than in a solid medium. Eight hearts were explanted into medium A, seven into medium B, eight into medium C.

Results—The results are best presented in a table summarizing the observations made on successive days. Table II shows for each day the number of hearts twittering, having co-ordinate beat, and stationary. An indication is given of the distribution within the hearts of both twitter and beat, by showing the numbers of cases in which these modes of behaviour occurred in both the major divisions of the heart (sino-atrial-ventricular region and conus), and in either of them but not in the other.

Table II

	Day 1 examination 1			Day 1 examination 2			Day 2 examination 1			Day 2 examination 2		
Medium	A	B	C	A	B	C	A	B	C	A	B	C
Twitter in both major divisions	5	5	5+1?	2	0	1 ?	2	5	1	1	5	1 (locatio unknow
Twitter in SA or V or both, not in conus	1	0	1	4	0	0	2	0	(1Td)	4	0	1
Twitter in conus only	2	1	0	0	3	0	0	1	0	0	0	0
Beat in both major divisions	0	0	1 trans	0	0	0	0	0	0	0	0	1(dislocat
Beat in SA or in both SA and V, not in conus	1	0	0	0	0	1	0	0	0	0	0	0
Beat in conus only	0	0	0	0	0	3	0	0	2	0	0	2
Stationary	0	1	1	2	4	3	4	1	5	3	2	4

Abbreviations, etc.—"Major divisions" are sino-atrial-ventricular region and conus. SA, sino-atrial regi
V, ventricle. Td, twitteroid. trans, transient.

A glance at the table shows that :—

(1) In the majority of explants in all three groups twittering occurred, in both the major divisions of the heart, immediately after explantation, while co-ordinate beating practically did not occur at that time. In the two hearts in which it did occur its presence was clearly due to the experimental medium not having had time to exert its full effect. In normal media beating always occurs at this stage.

(2) Setting aside the pre-incubation twitter, which is probably in large part a shock effect caused by the change of medium, the onset of twittering was earlier in medium A than in either of the other two groups (six hearts at the second examination on the first day, against three in medium B and perhaps one in medium C).

(3) The lowest concentration of potassium which caused twittering at all on the first day (medium B), caused it in the conus and not elsewhere, while the higher concentration (medium A) caused it more frequently in the atrio-

ventricular region. Co-ordinate beating occurred only in medium C and was usually confined to the conus.

(4) On the second day, twittering remained exiguous in medium C, but in medium B the amount of twittering had greatly increased and was now no longer confined to the conus, being present in both atria in four and possibly five cases, in the convex (apical) side of the ventricle in four and in all in the conus, particularly near its end. The concave side of the ventricle was in all cases stationary. In medium A twittering had somewhat decreased, but was still much more prominent than in medium C, and showed a preference for the sino-atrial and ventricular regions rather than for the conus. Co-ordinate beating never occurred in either medium A or B.

(5) It is concluded that:—

(*a*) The constituent of Sal EW which inhibits the co-ordinate beat and is responsible for the development of twitter is potassium, for excess potassium stops the beat and further excess produces twitter. Hence the agent responsible for the production of twitter in egg white must be its high potassium content.

(*b*) So far as the co-ordinate beat is concerned, the conus is more resistant to excess potassium than the rest of the heart.

(*c*) Twittering is usually induced in the conus by a lower concentration of potassium than is required to cause it in other regions.

Experiment 13—Besides its high content of potassium, the ash of egg white and the solution Sal EW contain relatively and absolutely less sodium than is present in balanced salines. The object of the present experiment was to see whether this low sodium content could be a factor in the production of twitter.

Four salines were used: (*a*) PC NaCl 0·3% was Pannett and Comptons' solution with the content of NaCl reduced to 0·3%, the amount present in Sal EW, the other salts being unaltered; (*b*) PC (0·352) was Pannett and Comptons' solution with the NaCl reduced to 0·3% and the other salts reduced in the same proportion. This solution was isotonic with NaCl 0·352%, and of course was slightly hypotonic to PC NaCl 0·3%; (*c*) NaCl 0·352%, with no other salts present; (*d*) NaCl 0·8% with no other salts present. The media consisted of nine parts of saline to one of plasma and are called medium A, B, C and D respectively. Fourteen hearts were explanted into medium A, twenty-one into medium B, eleven into medium C, three into medium D.

Results—(1) At the first examination, immediately after explantation, all three explants in medium D were beating and none were twittering, but in media A, B and C none were beating and nearly all showed some degree of twitter. The following table compares the three groups in respect of the number of cases of twittering in the major regions of the heart and of the activity of twittering.

Table III

	Medium		
	A	B	C
Number of explantsi n which—	%	%	%
Some degree of twitter occurred in SA	13 = 93	17 = 81	4 = 36
,, ,, AVC	9 = 64	10 = 48	1 = 9
,, ,, V	1 = 7	1 = 5	0
,, ,, conus	11 = 79	17 = 81	8 = 73
Twitter occurred in both atria (instead of only in one)	10 = 71	13 = 62	
Twitter was active in SA	5 = 36	5 = 24	0
Twitter was weak in SA	2 = 14	6 = 29	4 = 36
Twitter was active in AVC	4 = 29	3 = 14	0
Twitter was weak in AVC	1 = 7	6 = 29	1 = 9
Twitter was active in the conus	1 = 7	2 = 10	0
Twitter was weak in the conus ..	11 = 79	12 = 57	8 = 73

Abbreviations, etc.—The figures in the right-hand column are percentages of the total number of explants in each medium. SA, sino-atrial region. AVC, atrio-ventricular canal.

Thus (1) in regard to mere incidence of twittering in the sino-atrial region and atrio-ventricular canal, the explants in medium A gave the highest figures, those in medium B the next highest, and those in medium C the lowest figures. In the ventricle and conus the difference between the three groups is probably insignificant. (2) The incidence of twittering in both atria, instead of in only one, is higher in medium A than in medium B; the data for medium C are in this respect insufficient, but there is little doubt that the actual incidence was low. (3) The activity of twittering tended, in the sino-atrial region and atrio-ventricular canal to be greater in medium A than in medium B, but in the conus to be greater in medium B than in medium A, while in medium C activity was in all regions less than in either of the other two media.

Medium C was isotonic with medium B; therefore, if the low osmotic pressure accounted for all the twittering in medium B there should have been the same amount of twittering in both media. Actually there was definitely more twittering in medium B. But medium B differs from medium C in having less sodium and more of the other cations; it may therefore be concluded that so far as explants in medium B twittered more than those in medium C this was due to its lower ratio of sodium to other cations.

Explants in medium A twittered more than those in medium B, at least in the sino-atrial region and atrio-ventricular canal. Medium A was slightly hypertonic to medium B, therefore this greater activity was not due to a lower osmotic pressure; in regard to cation ratio it differed from medium B in the same sense as that in which medium B differed from medium C. Thus its greater activity in these regions must have been due to the same cause, that is, to a lower ratio of sodium to other cations.

Explants were made in medium D merely to show that a high ratio of sodium to other cations does not cause twittering.

Ordinary Pannett and Comptons' solution, which has the same cation ratio as PC 0·352 does not cause twittering. This fact has its explanation, which will be given in another paper.

(2) At the second examination on the day of explantation, and on succeeding days, the explants of all groups were beating. Twittering did not occur, after the first examination, in any explant in media B, C, or D, but it occurred in at least five explants, and probably in one other, in medium A. The twittering was never extended over the entire heart or even over a large part of it, but was usually restricted to quite small regions. Most frequently it occurred shortly before the death of the explant when the co-ordinate beat was absent or much weakened. Sometimes it occurred earlier, as in two explants on the second and third days respectively, when it appeared in the conus in the intervals between co-ordinate beats. No particular part of the heart appeared to be favoured, but the recognition of morphological regions was often difficult in late stages, owing to the spreading of the explants. Where it appeared early in the life of the explant it lasted for one to two or three days and then disappeared, while the co-ordinate beat might continue.

In media C and D, in both of which the ratio of sodium to other cations was high, the vigour, regularity and period of continuation of beating were in no way inferior, but rather better than in the other media. In medium C the beat persisted for periods ranging from two days in one heart to over two weeks in several.

It is concluded that:

(1) A low ratio of sodium to other cations favours twittering, and therefore the appearance of twittering in egg white is assisted by its low sodium content.

(2) Low osmotic pressures, considerably below that of egg white, can cause a transient twitter.

Experiment 14—It was stated in a footnote on a previous page that in the preparation of the solution Sal EW a mistake was made, the solution being made to contain 0·03% $CaCl_2 6H_2O$, whereas it should have contained twice this amount. The present experiment was intended to show whether the addition of the extra amount of calcium would affect the twitter-producing properties of the solution. Further, since it is not improbable that egg white after four or five days incubation of the egg may contain significantly more calcium than is present before incubation, I prepared a solution (Sal EW $CaCl_2 6H_2O$ 0·1%) having a higher calcium content than that supposed to represent the calcium content of the unincubated egg.

Three salines were used: one was Sal EW having as usual $CaCl_2 6H_2O$ 0·03%, the second and third resembled the first except that they contained 0·06% and 0·1% $CaCl_2 6H_2O$ respectively. Each saline was mixed with plasma in the proportion of nine of saline to one of plasma and the resulting mixtures were the media, which were used as solid clots. The mixture with Sal EW $CaCl_2 6H_2O$ 0·1% was called medium A, that with Sal EW $CaCl_2 6H_2O$ 0·06% was called medium B, that with Sal EW $CaCl_2 6H_2O$ 0·03% was called medium C. Eight hearts were explanted into medium A, nine into medium B, nine into medium C. All explants were examined immediately after explantation, again later on the same day, and thereafter daily.

Results—The accompanying table indicates the results over the first and second days. An arbitrary, and unavoidably somewhat subjective distinction has been made between active and weak twitters, the former indicated in Table IV by T, the latter by *t*. The distinction represents such differences as between a twitter affecting, for example, the whole sino-atrial region (T), and one confined to a single atrium with less active movement (*t*), or between a conus in which all or most of the cells are constantly contracting and relaxing and one in which an occasional contraction can be seen in odd cells here and there. Intermediate conditions, described in the notes by such terms as "moderate twitter," are included under T, so that the group *t* is composed of definitely weak twitters. Owing to the impossibility of measuring twitter, either by frequency of contraction, number of cells contracting, or by any other method, while some distinction such as that made is essential to the description of the facts, this rather arbitrary classification must be used. There is no doubt that it does give a fairly accurate representation of the differences in the effects of the three media.

Table IV shows that at the first examination, immediately following explanation, there was no very marked difference between the three groups; a slight inactivity in medium B, compared to those in media A and C, must surely be insignificant. At the next examination, the second on the day of explanation, the activity of all groups has decreased, and in medium C more than in media A and B, the only twitters existing in the former medium being in the conus and weak, while both the other two groups showed twitters in the sino-atrial region as well as in the conus. On the second day the greater activity in media A and B was well established, and four of each group now showed active twittering in the ventricle. In each of these eight explants twittering was present through the whole, or at least the greater part, of the sino-atrial region, along the convex (apical) side of the ventricle, and in the conus; that is, it was distributed throughout practically the whole heart except the concave side of the ventricle. In medium C no twittering occurred in the ventricle.

Table IV

	Day 1 examination 1			Day 1 examination 2			Day 2		
	Medium			Medium			Medium		
	A	B	C	A	B	C	A	B	C
SA	6 T 2 *t*	3 T 4 *t* 1 ?	6 T 3 *t*	4 T 2 *t* ?	1 T 4 *t*	0	6 T 1 *t*	7 T 2 *t*	1 T 2 *t*
Ventricle	2 *t* 1 T ?	0	2 *t*	0	0	0	4 T	4 T	0
Conus	3+1 ? T 4 *t*	4 T 4 *t*	5 T 3 *t*	2+2 ? T 3 *t*	5 T 3 *t*	7 *t*	4 T 3 *t*	6 T 3 *t*	3 T 3 *t* 1 ?

Abbreviations—SA, sino-atrial region. T, active or moderate twitter. *t*, weak twitter.

After the second day, the superior activity and extension over the heart of twittering in media A and B were maintained, and there is no need to describe the results further. Also the low nutritional quality of the medium began to make itself felt.

The osmotic pressure of medium C was, of course, slightly below that of media A and B, and it may be thought that this influenced the results. But the difference between the media B and C was only 0·03% $CaCl_2 6H_2O$, osmotically equivalent to about 0·01% NaCl. Further, experiment 13 showed that so far as osmotic pressure influences it at all, hypotonicity should favour the development of twitter.

The present experiment has been done twice. The results obtained on the first occasion have not been described because the number of explants in medium A was thought to be too small. Essentially similar results were obtained on both occasions.

The added calcium in media A and B both increased the activity of twittering and extended its range over the heart, particularly into the sino-atrial region;

it is concluded that, in egg white, twittering is due not only to the action of potassium in a medium of low sodium content but also to the action of calcium.

DISCUSSION

The object of the present work was to discover the cause of twittering in egg white, not to present a full investigation of the nature of this phenomenon or of all the factors influencing its production. The experiments described have made it clear that in egg white the principal factor responsible for the production of twitter is the high potassium content, and that this is aided by the calcium and is at least in part dependent upon the low content of sodium. It is possible of course, that the part actually played by calcium may be greater than the available facts would indicate, for the solution Sal EW is based upon the ash of unincubated egg white, not upon any knowledge of the actual free cations of egg white after four or five days incubation.

The present communication is not the first occasion upon which the phenomenon with which it deals has been reported. Olivo (1924) described the effects of treating explanted fragments of chick embryo hearts with excess calcium and potassium. He found, with both, an effect exactly similar to that which I have described, except that in calcium twitter (called by him " contrazioni dissociate ") might be accompanied by co-ordinate beating, an observation which I have since fully confirmed. In potassium, however, he found that the abnormal activity ceased after at most a few hours and could not be reawakened. This result is very different from mine; it is shown in this paper, and repeatedly in experiments which have not yet been reported, that twittering caused by potassium may continue for days. These divergent results may be due to differences in technique or to the fact that the material used by Olivo was usually derived from older embryos than those which I have used. It is a fact that, some hours after explantation, the preliminary twittering seen in my experiments immediately after explantation, and which appeared to be of the nature of a shock effect, tended to disappear; later, however, it usually reappeared and then continued until the death of the explant. I suggest, therefore, that the twittering which occurred in Olivo's experiments with potassium was this preliminary shock effect twittering and that the concentrations of potassium in his media were too low to produce the more permanent twitter. Olivo's other observations will be discussed in a succeeding communication. Bucciardi and Bisceglie (1929) also saw dissociated (twitter) contractions in excess calcium and excess potassium, but they did not investigate the phenomenon exhaustively.

The relation between twitter, co-ordinated twitter, twitteroid and co-ordinate beat presents a difficult problem which will not receive its full elucidation until further experiments have been made. A word may nevertheless be said on co-ordinated twitter and twitteroid. The characters of twitter are: (*a*) lack of co-ordination between the cells; (*b*) small amplitude of the contractions; (*c*) the contractions are repetitive but arhythmic, that is, no regularity can be discovered in the lengths of the intervals between contractions; (*d*) the frequency of contraction of each cell is greater than the frequency of the intrinsic co-ordinate beat of the region of the heart concerned, and is greater in the sino-atrial region than in the ventricle, and in the ventricle than in the conus. Co-ordinated twitter appears to be a condition in which each cell contracts in the manner seen in ordinary twitter, but with more or less co-ordination, while in twitteroid there is a mixture of twitter with a co-ordinate beat whose co-ordination has been rendered imperfect by the same factors as have caused the twitter.

The fundamental problem of the relation between fibrillation as seen in the mammalian heart, co-ordinate beat and twitter will be discussed on another occasion after the presentation of further data.

In conclusion, it is a pleasure to express my thanks to Dr. H. B. Fell for assistance given in many ways, to Mr. C. W. Wilson who helped with the p_H determinations, and to my wife who prepared many of the explants.

SUMMARY

1—Fragments of chick embryos in primitive streak stages, taken from parts of the embryo known to contain the heart anlagen, and explanted into egg white, survived as vesicular structures in which a degree of differentiation might occur. In egg white of eggs incubated for four or five days areas of contracting tissue appeared, but not in egg white from eggs incubated for two days or less.

2—Similar fragments, explanted into plasma and embryo extract, produced areas of contracting tissue; the contractions were rhythmic and co-ordinate. In egg white the cells contracted in a quite anarchic manner without co-ordination and arhythmically; the contractions could continue for long

periods of time, in one intance for 36 days, although the medium was not changed.

3—The unco-ordinated contractions superficially resembled fibrillation, but as identity with fibrillation remains doubtful, the present phenomenon is given the provisional name " twitter."

4—The entire hearts of embryos incubated for two or three days behaved similarly when explanted into egg white. Egg white from eggs incubated for less than two days was not tried; in egg white from eggs incubated for two days twittering occurred in one explant, the hearts being stationary in the remainder, but in egg white from eggs incubated for four or five days, while the co-ordinate beat stopped in all, it was frequently replaced by twitter. Five day egg white was a more active agent in producing this phenomenon than four day egg white. Older egg whites were not tried.

5—The cause of twittering was investigated, mainly by the use of a saline whose content of sodium, potassium, calcium, magnesium resembled that of the ash of egg white. It was found that the principal agent in the causation of twitter in egg white is the high content of potassium,that this is aided by the much lower, but still rather high content of calcium, and that the occurrence of twitter is assisted by the low content of sodium.

6—The co-ordinate beat was more susceptible to excess potassium in the sino-atrial region than in the conus, so that it might persist in the conus in a content of potassium which had stopped it elsewhere. The frequency of beat in the conus was then low, resembling the intrinsic beat of the same region when mechanically isolated from the rest of the heart.

7—Twittering was usually induced in the conus by a lower concentration of potassium than was required to cause it in other regions.

8—Osmotic pressures much below that of egg white produced a slight transient twitter which was soon replaced by a co-ordinate beat which could be maintained for days. The osmotic pressure of egg white is too high to be a cause of twitter.

9—The characters of twitter are: (*a*) absence of co-ordination between the contracting cells; (*b*) short amplitude of contractions; (*c*) the contractions are repetitive but arhythmic; (*d*) the frequency of contraction of any cell is greater than the intrinsic frequency of the co-ordinate beat in the region of the heart in which the cell lies; (*e*) the frequency of contraction is very great in the sino-atrial region, less in the ventricle, least in the conus; (*f*) twittering may occur distributed over all or most parts of the heart or may be restricted to almost any large or small region in it.

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Hypophysectomy of Birds. I—Technique, with a Note on Results

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[Plate 16]

I—*Introduction*

Hypophysectomy is now performed readily in many mammals and amphibians, but so far it has apparently not been successfully accomplished in birds. Fischera (1905) attempted to cauterize the pituitary body of the fowl, but obtained only dubious results. In view of our increasing knowledge of the reproductive system and secondary sexual characters of birds, it is evident that hypophysectomy would open up a wide field of investigation for work on sex hormones. The parapharyngeal route for removing the pituitary was precluded by the large size of the gullet, while the temporal route seemed to offer little hope. The extensibility of the mouth tissues, however, suggested that a buccal approach would be practicable. It was found barely possible to open the beak sufficiently to give easy access to the back of the mouth, but a comparatively small incision in the thin elastic floor of the mouth provided a ready approach to the soft palate and the base of the skull. We have found it possible to remove the pituitary with comparative ease by this transbuccal route, and the present paper is devoted to a description of the exact technique of the operation.

II—*Operative Technique in the Fowl*

The following description applies primarily to the fowl; notes on the differences found in other birds are given in Section III.

The bird is given a small sub-cutaneous dose of urethane (about 0·5 gm per kg) and a large dose of atropine (about 20 mg) and anæsthetized with ether. This amount of urethane does not retard recovery appreciably and makes it easier to give the ether. Atropine is essential to control the copious mucous secretion in the mouth.

The ventral surface of the neck is plucked from the sternum upwards. A U-shaped tracheal cannula is inserted low down in the neck, each arm of the tube being about 5 cm long, and a piece of rubber tubing is then attached to the free end to lead back over the breast to the anæsthetist. The normal airway is not completely occluded in the operation on the fowl, but the use of a tracheal cannula, though not essential, is highly convenient. The bird is placed on its back, head towards the operator. The head is kept vertical by an adjustable and suitably shaped block on each side and is held down by an elastic band over the beak. Between the blocks a slit is cut in the board to accommodate the comb.

An incision about 2 cm long is made through the skin and platysma between the wattles, starting about 1·5 cm behind their anterior margin, fig. 1. If the wattles are very much developed and fleshy they may meet in the mid-line, and it is then necessary to make the incision farther back and to retract the skin anteriorly. Interference with the complex of blood vessels at the base of the wattles may lead to their becoming gangrenous and must always be avoided. The edges of the skin are retracted by small hooks attached to elastic. The anterior part of the hyoid apparatus and the thin muscles covering the floor of the mouth are now in view. By pushing the hyoid bones to the left, a strip of the floor of the mouth free from muscle can be seen in the angle between the mylo-hyoid and the stylo-hyoid muscles. An incision about 2 cm long running anterio-posteriorly is made at the right-hand margin of this area. The incision must be kept well to the right, otherwise little tissue will be left against the base of the tongue and the subsequent suturing will be more difficult. The tongue and hyoid apparatus are now retracted to the left by further hooks and a slight retraction on the right-hand margin and anterior end of the incision completes this stage of the operation. The glottis is about 3 cm posterior to the incision and is unaffected by these manipulations.

Part of the hard palate* and almost the whole of the soft palate are now in view. The latter is deeply cleft, except in the middle, where a bridge of tissue connects the two sides at a point above the articulation of the pterygoids and palatines with the rostrum, fig. 2.

Since there is no nasal pharynx in the fowl, access to the base of the skull is easy. The ridge forming the anterior extremity of the basi-temporal bone† can be felt with a seeker below the posterior end of the cleft in the palate. The narrow rostrum can also be felt under the bottom of the cleft and can be traced forwards to its junction with the vomer which projects into the much deeper cleft in the hard palate. The posterior part of the cleft in the soft palate is deepened by blunt dissection and the two sides of the palate are pushed laterally and held in position by a spring retractor of the type used by

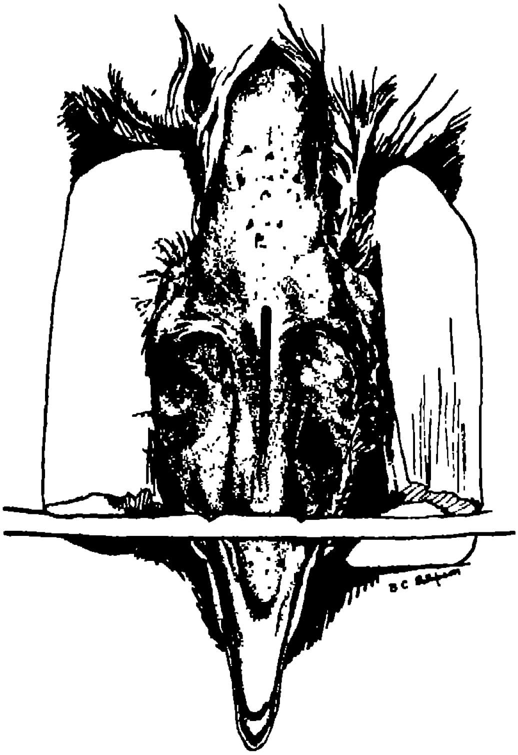

FIG. 1—Ventral surface of head of fowl, showing line of skin incision.

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* "Hard and soft palate" are here used as descriptive terms for regions not homologous with those so called in mammals.

† The terminology used is that of Parker (1869).

White (1933) for the rabbit. The rostrum is most easily cleared of connective tissue and periosteum by light cauterization. The cavity thus made is bounded laterally by the retracted palate, posteriorly by the crest of the basi-temporal, and anteriorly by the bridge of tissue across the cleft of the palate, fig. 3. The openings of the Eustachian tubes are at the posterior end of the rostrum, and are obliterated by the operation. An oval hole about 3 mm by 5 mm is now drilled in the posterior rostrum with a No. 13 dental burr, the posterior margin of the hole extending to the basi-temporal. The rostrum is quite narrow and unless the hole is made exactly in the mid-line, it is easy to get lost in the soft tissues at the side. Before drilling is begun it is advisable to make sure that the preliminary manipulations have not pushed the head out of the vertical, as loss of direction in drilling is much more likely if the head is leaning to one side. The rostrum is soft and "honeycombed," but little bleeding should occur during drilling. When the hole is about 3 mm deep, the pituitary comes into view. Approached from this direction, the pituitary lies at an

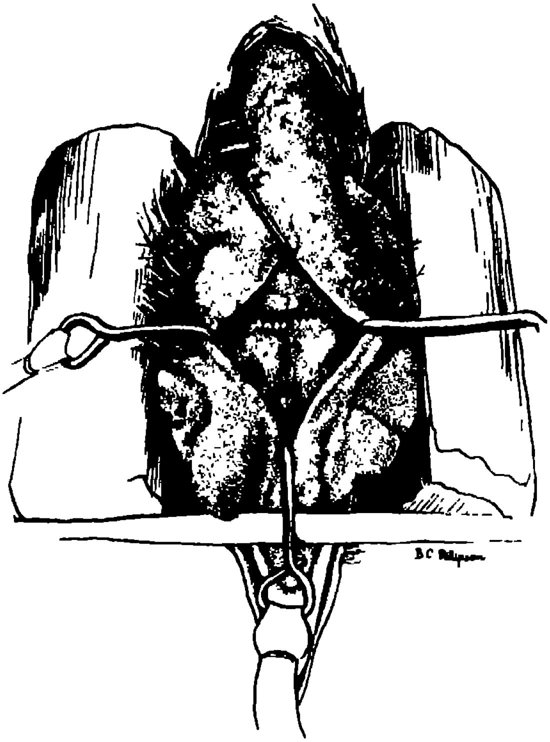


FIG. 2—First stage of hypophysectomy : floor of mouth opened, and palate exposed.

angle, and the posterior portion is seen first. Attention is then directed to deepening the anterior part of the drill hole and exposing the remainder of the pituitary, which is comparatively large in area, about 2 mm by 4 mm, but quite thin. As in mammals, the last thin plate of bone should be picked out with needle and forceps. The pituitary is surrounded by sinuses, and the internal carotids are almost embedded in the sides (see de Beer, 1926), so that excessive enlargement of the drill hole, especially laterally, leads to serious bleeding. The posterior lobe is small, and from the operative approach lies under the posterior part of the anterior lobe, fig. 6, Plate 16. According to de Beer there is no pars intermedia, and only a very thin pars tuberalis covering the stalk. When the last plate of bone is removed at the bottom of the drill hole, the pituitary bulges up inside its capsule, fig. 4, which is remarkably tough and is best divided by tearing down in the mid-line with a sharp hooked needle. The pituitary can then be removed by a negative pressure cannula. Usually the whole gland comes out in one piece, but sometimes it is necessary to clean up fragments adhering to the stalk. Except for the connecting stalk the pituitary is entirely cut off from the brain. The whole of the posterior lobe and about one-third of the anterior lobe is covered by the dorsum sellæ, fig. 6, Plate 16, and the remainder is separated from the cranial cavity by a tough membrane. Even this is not in contact with the brain, as the optic chiasma is very large and lies right above the anterior part of the sella turcica. The risk of injury to brain tissue during the removal of the pituitary is slight.

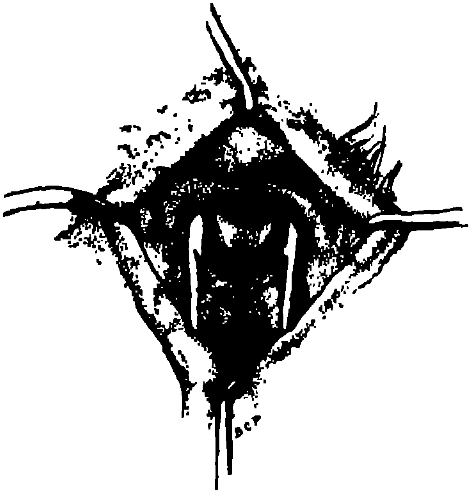


FIG. 3—Second stage of hypophysectomy: palate retracted laterally and rostrum exposed.

When the removal is completed the dorsum sellæ should be clearly visible as a floor to the posterior half of the cavity. Appreciable bleeding may occur when the gland is sucked out, and we have usually plugged the sella turcica and drill hole with shredded gut so that the operation can be proceeded with immediately. The plug is partially reabsorbed in the course of time and appears to have no ill-effect. Removal of the spring retractor allows the two halves of the palate to slip back over the drill hole and the operation is completed by suturing the incision in the floor of the mouth, removing the cannula and closing the tracheal and skin wounds. The well-known resistance of birds to infection makes it possible to carry out the whole operation with a minimum of aseptic precautions.

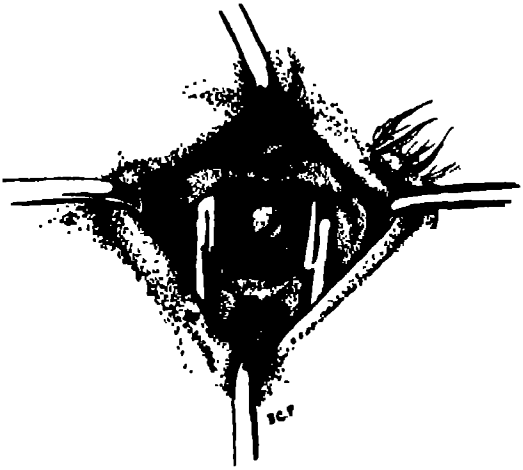


Fig. 4—Third stage of hypophysectomy: rostrum drilled and pituitary exposed.

III—*Recovery and Effects*

The fowl recovers rapidly from the anæsthetic and is walking about and eating within an hour. It usually appears to feel no after-effects from the actual manipulation. The skin wounds heal with remarkable rapidity. Some few birds have developed mucous obstruction in the glottis, but how far this is an effect of the manipulation and how far an effect of the remarkable debility which follows the operation is difficult to say. For 12–18 hours the birds behave normally, but subsequently severe metabolic disturbances set in and unless temporary replacement therapy is begun immediately the great majority die within 48 hours. Anterior lobe extracts, or to a lesser degree cortin, assist the birds through the critical period. We are thus satisfied that the mortality following the operation is not due to operative shock or other casual factors. In birds kept alive, the changes which might be expected occur in the reproductive tract and secondary sexual characters. Thus the testes of the cock atrophy, and the comb regresses in the same way as after castration. The ovary and oviduct of the hen also undergo involution. Plumage changes, referable to atrophy of the gonads and thyroid, occur in both sexes. These effects will be dealt with later.

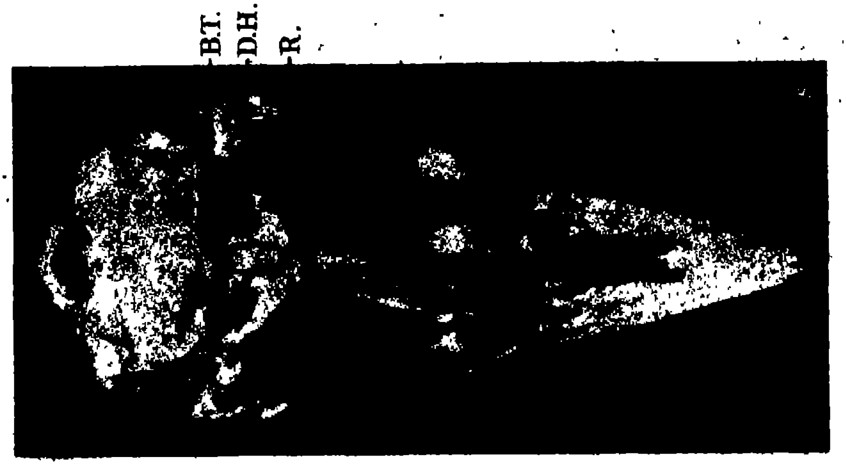
IV—*Adaptation of Operative Technique to Birds other than Fowls*

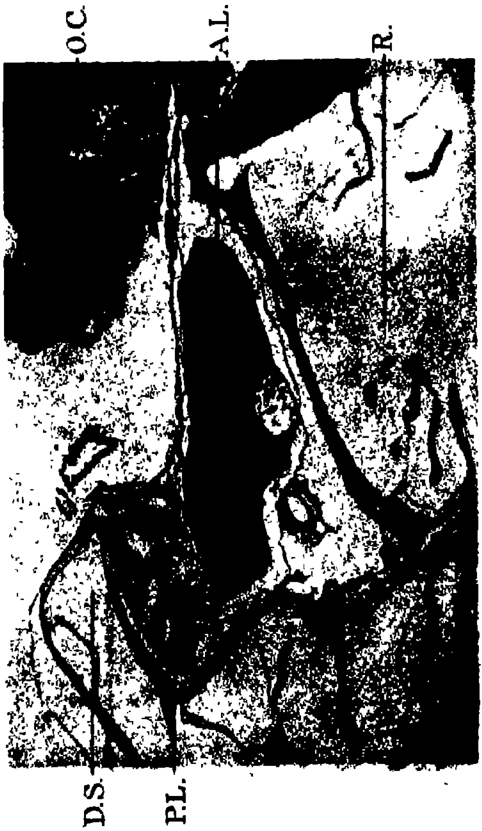
We have investigated the possibility of performing a similar operation on other birds, particularly bantams, ducks, pigeons and doves, and turkeys. With certain slight modifications due to minor differences in the morphology of the area, the operation can be adapted to these other species and has been successfully performed on them.

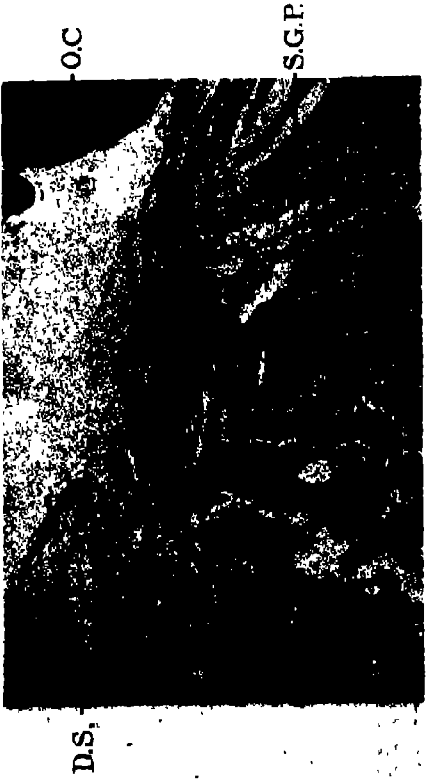
Bantam—Exactly the same technique is required for the bantam, except that a smaller spring retractor and a No. 10 dental burr are required.

Duck—The longer, narrower head of the duck makes the area of operation rather more cramped. Further, the trachea comes right up between the wings of the hyoid bone and the glottis lies at the base of the tongue, which is much larger than in the fowl. In spite of the greater congestion of the hyoid region, however, the floor of the mouth can still be retracted to expose the soft palate. The glottis, however, is occluded and a tracheotomy is essential in this species. The soft palate is more deeply cleft than in the fowl, and is V-shaped in section, so that the rostrum is nearer the surface and can be exposed with ease. It is broader and much longer than in the fowl. The pituitary is situated slightly further forward in relation to the basi-temporal crest than in the fowl, but otherwise there are no differences in the rest of the procedure.

Pigeon and Dove—The essential topography is much the same as in the fowl. The basi-temporal crest is nothing like so prominent, but if the anterior part of the cleft in the soft palate is enlarged and the palate spread laterally the proper place for drilling the rostrum is reached. The bone is even more honeycombed and is rather thicker than in the fowl, and the pituitary itself is relatively larger, but no essential difference in technique is required. The smaller size of the head necessitates the use of a much smaller spring retractor than is used for the fowl and a No. 8 dental burr is required for drilling.







(*Facing p.* 400.)

Turkey—The topography of the turkey head is also very similar to that of the fowl, except that the glottis is nearer to the tongue. The operative procedure is identical, but the site is much more roomy and larger instruments are required. A No. 16 dental burr can be handled with ease.

V—*Summary*

1—A technique for transbuccal hypophysectomy of the fowl is described, together with the necessary modifications for other birds.

2—Hypophysectomy in the fowl usually results in death within 48 hours. This effect, which can often be avoided by appropriate replacement therapy, is apparently due to the severe metabolic disturbance which follows removal of the pituitary.

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DESCRIPTION OF PLATE

Guide Letters

a.l., anterior lobe of pituitary. *b.t.*, basi-temporal crest. *d.h.*, site of drill hole for hypophysectomy. *d.s.*, dorsum sellæ. *o.c.*, optic chiasma. *p.l.*, posterior lobe of pituitary. *r.*, rostrum. *s.g.p.*, shredded gut plug.

FIG. 5—Ventral surface of skull of fowl, lower jaw removed, showing site of drill hole for hypophysectomy.

FIG. 6—Sagittal section of sella turcica and surrounding region in the fowl. × 12.

FIG. 7—Section of same region 5 days after hypophysectomy, showing the shredded gut with which the drill hole and sella was plugged. In this case a small piece of posterior lobe is still present under the dorsum sellae, but no anterior lobe tissue. × 12.

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Induction of Fertility and Pregnancy in the Anœstrous Ferret

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[Plates 17 and 18]

I—*Introduction*

Bissonnette (1932) was able to induce complete œstrus in three female ferrets by evening illumination from October 12 onwards, but similar treatment of a male from October 12 to December 22 failed to carry spermatogenesis beyond the spermatocyte stage. Mating, therefore, resulted only in pseudopregnancy. In a second series of animals illuminated from December 7 onwards, spermatogenesis was secured during February and a litter was born on April 11 (Bissonnette, 1933). The normal untreated male, however, usually becomes fertile during February (Allanson, 1932) and the first litters of the season may be born early in April, so that Bissonnette cannot be said to have induced fertility outside the limits of the normal breeding season.

Bissonnette's failure to bring about premature spermatogenesis, though involving one animal only, suggested that the male was much more refractory than the female, especially since anterior lobe extracts and urine of pregnancy extracts readily cause ovulation in the anœstrous ovary (Hill and Parkes, 1930), while such extracts cause little reaction in the quiescent winter testis. It seemed, however, that a combination of additional light and gonad-stimulating extracts might be effective, and the present paper records experiments carried out on these lines, as a result of which pregnancy was induced in light-treated females well outside the limits of the normal breeding season.

II—*Technique and Material*

Illumination—The evening illumination was carried out exactly as described in a previous paper (Hill and Parkes, 1933, *a*).

Gonad-stimulating Extract—An extract of urine of pregnancy (U.P. extract, B.8), kindly prepared by the British Drug Houses, was used. This had a rabbit unit (see Hill, Parkes and White, 1934) of about 0·5 mg. It was kept as a dry powder and sterilized by heat (see Askow and Parkes, 1933) before being made up in sterile saline for injection.

Histological Technique—This was as usual; the organs were weighed at the 70% alcohol stage during up-grading. Since three testes and epididymides were obtained by unilateral castrations, the weight of organ given is for one only, or is the mean weight if the pair were obtained together. The ovary weight given is that of the pair.

Scheme of Experiment—Twelve females (FPL 1–12) were put on to evening illumination on October 12. Eleven of these came into œstrus during the next three months, 9 being used for the work recorded in this paper and 2 for other purposes. Four males (FPL 13–16) were put on light treatment at the same time; two stages were obtained from each of three of these males by means of unilateral castration. Three normal males (FPL 18–20) were killed as controls, and a further one (FPL 17) was injected with urine of pregnancy extract.

III—*Activation of the Female*

The Normal Anœstrous Female—The condition of the reproductive organs of the normal female ferret during the winter has been described by Hammond and Marshall (1930) and some additional information was given by Hill and Parkes (1933, *b*). Hammond and Marshall describe their earliest full œstrus as occurring on March 2. The earliest œstrus in our experience was seen on February 20. Major Dunkin informs us that for the three years 1933, 1932 and 1929, the earliest dates of mating in the large colony maintained at the National Institute's Field Laboratories have been February 10, March 9 and April 3, respectively.

Activation by Light—Of the twelve females put on to light from October 12, one reacted in 5 weeks, one more within 8 weeks, eight of the remainder within 3 months, and one more in a little over 3 months. One failed to show the vulval swelling even after 5 months. Eight of the females were mated and they all ovulated, although the duration of mating was very short in some, being as little as 10 minutes in one. There is some evidence that these light-treated females were stimulated to supernormal ovarian activity. As shown in Table II, six ferrets, pseudopregnant from copulation at light-induced œstrus, had a total of 80 corpora lutea, an average of over 13 per animal. One animal had 20 and another 17. The numbers of corpora lutea in these animals are certainly above the average size of litter (8–9) and probably above the average number of follicles ovulating at one time. The economic possibilities of this apparent increase in female fertility, however, are not great, because the average litter size in normal ferrets is obviously approaching the maximum number of young which could be carried to full term and reared normally.

IV—*Activation of the Male*

The Normal Anœstrous Testis and Epididymis—The anœstrus of the male ferret can be divided into two phases (Allanson, 1932); one of almost complete inactivity (October–November), and another of gradual preparation for the breeding season (December–February).

In October and November the weight of a single testis is 0·16–0·20 gm, the seminiferous tubules measure 75–100 μ in diameter and have a prepubertal appearance. There is no active production of spermatocytes, although they are found in occasional tubules. In December primary spermatocytes are more numerous and arranged in regular rows two or three cells deep around a small lumen. The diameter of the tubules has increased slightly to 120–140 μ. In January spermatid production begins and a few tubules in a section show an enlarged lumen surrounded by several rows of spermatids. Early in February elongating spermatids are found, and later in the month mature spermatozoa are produced. From March to July the weight of one testis is 1·25–2·0 gm.

The intertubular elements of the testis are most reduced during October and November; the interstitial cells are small (110–120 sq μ) and their cytoplasm contains few granules and stains faintly with eosin. During the preparation for breeding activity the interstitial cells gradually enlarge, their cytoplasm becomes more densely granular and stains intensely, and fats and lipoids accumulate. The maximum glandular development is reached when ripe spermatozoa are appearing in the seminiferous tubules, *i.e.*, in February, although the accumulation of fat continues, so that the cells increase somewhat in size until the middle of the breeding season.

The weight of the epididymis during anœstrus varies up to 0·150 gm, while the diameter of the tubule is 80–110 μ.

Effect of Urine of Pregnancy Extract Alone—In our experience anterior pituitary or urine of pregnancy extracts have consistently failed to activate the atrophic testis of the anœstrous or hypophysectomized male ferret. This is shown in the present material by FPL 17 (see Table I), which was given 5 mg U.P. extract per day from December 20–December 29, and in which the testes were actually more atrophic than those of the untreated male killed two days later (FPL 18). The seminiferous tubules in FPL 17 were small and a large proportion contained only spermatogonia and Sertoli cells, but unlike the normal tubules of this type, there was a large empty lumen, fig. 2, Plate 17. The remaining tubules contained fairly numerous spermatocytes, which were scattered and irregular. It would appear that the injections had actually retarded the development of the testis since it had remained in much the same condition as in the depths of ancœstrus, and the empty vacuolated tubules gave an added appearance of regression. The interstitial cells showed no change from the ancœstrous condition.

Effect of Light Alone—The left testes of two ferrets (FPL 14 and 15) put on light on October 12 were removed on November 28 after 47 days treatment. They were much larger (see Table I) than normal ones at this time of year. The seminiferous tubules were somewhat larger (136–150 μ), but individual variation in diameter was greater than in a normal testis. The spermatogenic activity also varied much from tubule to tubule, fig. 3, Plate 17. The majority of tubules contained numerous primary spermatocytes but a few were lined only by a single row of cells, Sertoli nuclei and spermatogonia. Occasional tubules, however, were producing spermatids, of which some were beginning to elongate. The testis, as a whole, therefore, is comparable to that of Bissonnette's ferret which had 71 days of light treatment.

The interstitial cells were strikingly different from the normal, having enlarged to breeding season size (288–335 sq μ); the compact islands lying between the half-developed seminiferous tubules were therefore much more conspicuous than the interstitial tissue in the normal testis at any period of the reproductive cycle. This development of the interstitial cells may have accounted for some of the increased weight of the organs (0·71 and 0·89 gm) which was high for the degree of activity of the seminiferous tubules. The observations correspond with those of Bissonnette.

The epididymis similarly showed advance over the normal histological condition, the tube being enlarged (133–145 μ) and the epithelial cells appearing more active.

FPL 13 was kept on light without other treatment till January 1, when it was killed after 81 days evening illumination, 10 days longer than Bissonnette's first male. During this period it had mated with 3 light-treated females, on December 18, 19, 22. Each of these females became pseudopregnant, although two of the copulations were very brief. When FPL 13 was killed on January 1 it was seen that the testes were large and vascular, and spermatozoa were found in a smear from the epididymis. Histological examination confirmed this observation. The seminiferous tubules were in spermatogenic activity and the majority contained spermatozoa, fig. 4, Plate 17, but production of mature spermatozoa could not have been proceeding for long, since they were present in large numbers only in the caput epididymis. This result agrees with the fact that the mating 10 days earlier was sterile. The islands of interstitial cells were less conspicuous than in the left testes of FPL 14 and 15, owing to the enlargement of the seminiferous tubules (171 μ) and the consequent return to the normal relative proportions of tubular and intertubular elements, but they were fully developed in size and glandular appearance. The epididymis was more advanced than in FPL 14 and 15, the tube measuring 163 μ in diameter, and the non-vibratile threads of the epithelium were fully developed.

Effect of Light and U.P. Extract Combined—Immediately after the left castration of FPL 14 and 15 injections of U.P. extract were started, 5 mg per day was given for 6 days, the evening illumination being continued, at the end of which period the animals were killed (December 4) and the right testes fixed. The right testes and epididymides of FPL 14 and 15 showed little advance in weight over the left organs examined after 47 days of light alone, but there was a definite increase in spermatogenic activity in the seminiferous tubules. Approximately half these contained several rows of spermatids which frequently were elongating to form spermatozoa, fig. 5, Plate 17. Although few mature spermatozoa were found in the testis, a small number had reached the upper coils of the epididymal tube in one of the animals, FPL 14. There had been no increase in the diameter of the seminiferous tubules, and the interstitial cells resembled those in the left testes.

If these results are compared with those recorded in the previous section, it is evident that the stimulation of the testis had been expedited by the U.P. extract. A third light-treated ferret (FPL 16) was therefore given U.P. extract. This animal, which was also put on light on October 12, had sterile matings with light-treated females on November 22, and December 4, and 13. Beginning on December 18, after 67 days on light, 5 mg was given for 10 days, the light treatment being maintained. On December 28, the day after the last injection, the right testis was removed. The weight was up to breeding season standard, and a vigorous suspension of spermatozoa was obtained from the epididymis. Histologically, all tubules of the testis were in active spermatogenesis, fig. 6, Plate 17, and spermatozoa were present in the epididymal tube throughout its entire length, and had passed into the upper part of the vas deferens. This testis, compared with those of FPL 13, shows that a definite acceleration of spermatogenesis had been produced by the U.P. extract. Considering this result in relation to the earlier ones described above, it appears that U.P. extract can only affect the spermatogenic tubules when they have reached a certain stage of spermatogenesis, probably when secondary spermatocytes have been produced, but not before. On the day following removal of the first testis, December 29, the male was mated with FPL 2, a light-treated female in full œstrus. Copulation was prolonged to 1 hour 20 minutes, and the female became pregnant.

On January 1 the male was mated to another light-treated female (FPL 7). Copulation lasted 2 hours 10 minutes, and a vigourous suspension of spermatozoa was obtained from the vagina. This female also became pregnant. The male was taken off light on January 4 and killed on January 16, when the left testis was still in full activity. Two more normal controls killed on January 12 (FPL 19 and 20) were still only showing the first signs of breeding season activity, fig. 7, Plate 17, and, Table I, and there can be no doubt that the induction of spermatogenesis in the four males during December must be attributed to the experimental treatment.

V—*Course of Pregnancy and Lactation*

The results described in Section VI were not available at the time and as it seemed probable that FPL 2 and 7 would become pregnant following the matings described in Section IV, they were kept on evening illumination. The vulvæ subsided normally during the fortnight after mating, and at 3 weeks fœtuses could be palpated in both animals. The subsequent course of pregnancy was uneventful. A few days before full term the animals were given bedding and proper accommodation for littering down. This, of course, reduced the amount of illumination during the evening. FPL 2 had a litter of 9 young on February 8 and FPL 7 one of 10 on February 11, the period of gestation being 41 days in both and therefore quite normal. FPL 2 suckled 4 of her litter well until dying of distemper on March 7, by which time the young were so advanced that rearing was completed by hand. FPL 7 suckled the whole of her litter until developing a mammary abscess on February 18, which necessitated the removal of the young. The abscess was cleaned up surgically, and the animal was in heat again 3 weeks later.

VI—*Effect of Terminating Extra Light after Ovulation*

Bissonnette's results made it evident that the usual pseudopregnant changes followed sterile copulation if the light treatment was continued. The work

Table I

Number of ferret	Date put on light (1933)	Date organ obtained (1933–34)	Days on light	Days on U.P. extract	Testis				Epididymis		
					Weight (1) (gm)	Mean tubule diameter (μ)	Stage of spermatogenesis	Area of interstitial cell (sq μ)	Weight (1) (gm)	Tube diameter (μ)	Tube contents
FPL 14 LT	Oct. 12	Nov. 28	47	—	0·710	150	SPT	335	0·192	133	—
FPL 15 LT	Oct. 12	Nov. 28	47	—	0·896	136	SPT	288	0·174	145	—
FPL 13	Oct. 12	Jan. 1	81	—	1·825	171	SPZ	439	0·352	163	SPZ caput
FPL 14 RT	Oct. 12	Dec. 4	53	6	0·796	154	SPZ few	452	0·252	137	SPZ few
FPL 15 RT	Oct. 12	Dec. 4	53	6	1·174	156	SPZ few	452	0·268	137	SPZ few
FPL 16 RT	Oct. 12	Dec. 28	77	10	1·480	194	SPZ	—	0·307	174	SPZ many
FPL 16 LT	Oct. 12	Jan. 16	96	10	1·350	187	SPZ	330	0·300	175	SPZ many
FPL 17	—	Dec. 30	—	10	0·286	113	SPC	207	0·097	127	—
FPL 18	—	Jan. 1	—	—	0·500	136	SPC	194	0·109	105	—
FPL 19	—	Jan. 12	—	—	0·434	138	SPC	181	0·096	121	—
FPL 20	—	Jan. 12	—	—	0·417	141	SPC	211	0·225	137	—

SPC = spermatocytes; SPT = spermatids; SPZ = spermatozoa.

recorded above showed that pregnancy and lactation would develop normally under these conditions. It seemed desirable, however, for both theoretical and practical reasons to find out whether continuance of evening light was essential for the occurrence of the typical post-ovulation changes after mating at induced œstrus.

Three females in induced œstrus were therefore mated and left on evening light; three others were mated and taken off evening light. All the matings were sterile. The lighted animals were killed at 17, 27 and 38 days p.c., and the unlighted ones at 14, 28 and 42 days p.c. The details are shown in Table II. Examination of the ovaries showed that the life history of the corpora lutea of those receiving extra light p.c. had been essentially normal, the full diameter of rather over 2 mm being attained at 4 weeks p.c. In the series receiving no extra light p.c. the corpora lutea were fully up to normal size at 2 and 4 weeks. At 6 weeks (FPL 4) the average size of the 17 corpora lutea in the two ovaries was 1·24 mm. Hammond and Marshall's figures for the largest corpus luteum at 5½, 6 and 7 weeks pseudopregnant are 1·65, 1·98 and 1·11 mm, respectively, and 1·33 mm at 6 weeks p.c., just after parturition. These figures obviously indicate a wide range of variation, and in view of this the corpora lutea of FPL 4, though small, cannot be considered abnormal.

Table II

No. of ferret	Date mated	Days on light at mating	Subsequent treatment	Days killed p.c.	No. of corpora lutea	Average diameter corpus luteum (mm)
FPL 9	December 13	62	Continued light p.c.	17	10	2·09
FPL 5	December 19	68	,,	27	10	2·14
FPL 3	November 22	41	,,	38	11	1·83
FPL 8	December 22	71	No extra light p.c.	14	12	2·07
FPL 11	December 18	67	,,	28	20	2·10
FPL 4	December 4	53	,,	42	17	1·24

Histological examination of the uteri pointed to the same conclusions. At 4 weeks p.c. the typical pseudopregnant proliferation in the uterus was well developed in both series, showing that the corpora lutea were both physiologically and morphologically normal in the absence of extra light p.c. The uterus of FPL 3, 38 days p.c., had obviously been very well developed, but regression was just setting in. FPL 4, 42 days p.c. without extra light, was far regressed from the pseudopregnant condition, a finding in keeping with the small size of the corpora lutea. The reproductive organs of this animal, however, cannot be considered outside the range of variation found normally at the end of pseudopregnancy. The mammary glands for both series showed the usual pseudopregnant condition. On the evidence of these six animals, therefore, it may be concluded that once the pituitary body has been stimulated by additional light, normal pseudopregnancy will follow ovulation without further treatment. In this connection, it may be noted that one light-treated female in full œstrus on January 16, was taken off extra light for 5 weeks without any change in the vulval swelling. Evidently, the pituitary remains activated for some time after the withdrawal of the stimulus of additional light.

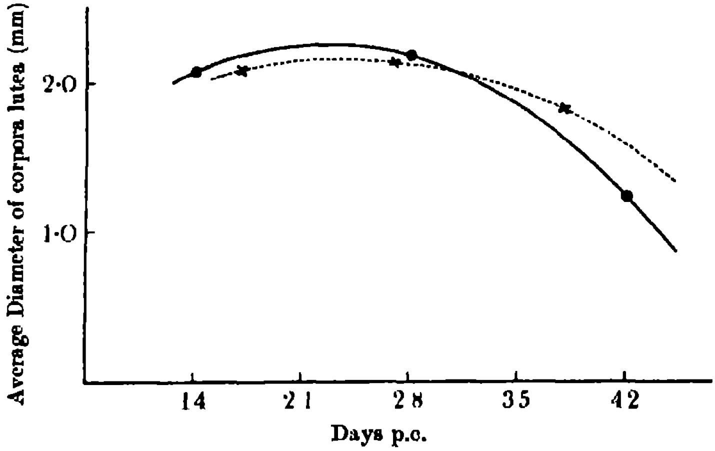

FIG. 1—Size of corpora lutea with and without extra light post-coitum. · · · · with extra light; ——— without extra light.

VII—*Discussion*

The experiments described above add little to our existing knowledge of the induction of œstrus by extra light in the quiescent female ferret, except to emphasize the individual variation in the length of the latent period and to suggest that an unusually large number of ripe follicles may be found at light-induced œstrus. Further results have been obtained, however, with the quiescent male. Full spermatogenesis was caused by extra light in 11½ weeks, and there can be little doubt that if Bissonnette had left his male on light another 2 weeks he would have attained the same result. The stimulating action of light was much augmented by administration of U.P. extract, the production of spermatozoa having started in one such dually treated animal in the first week of December. It seems probable that the breeding season of ferrets may now be greatly extended.

With a gestation period of 6 weeks, an essential lactating period of at least 6 weeks, and a pro-œstrus of 2–3 weeks, the minimum time for a complete cycle is nearly 4 months, so that the rearing of three litters per year is about the most that can be hoped for from a single female. This should be quite practicable.

Since ferrets normally cease to breed early in August when there is still 17–18 hours' daylight, it is not clear how they could be kept in breeding condition during August and September. On the other hand, females getting pregnant in July or early in August carry and suckle their young quite normally, although the latter are not ready for weaning till the end of October. Such females could be brought into œstrus again and mated by the end of the year. Their first litter of the season would therefore be born in the middle of February and weaned early in April. By the end of April they would be in œstrus and pregnant again, the second litter being born early in June and weaned towards the end of July. This would enable them to come into œstrus again naturally just before the end of the normal breeding season. On this scheme, males put on to light at the beginning of October, would be fertile and ready for mating at the end of the year, and U.P. extract treatment would be unnecessary—a practical convenience for the breeder. On the other hand, females which failed to get pregnant again at the end of the normal breeding season could be put on light towards the end of September, brought into œstrus during November and mated with males given U.P. preparations after 6 weeks light. The same technique should be possible with young females born early in the year. The only possible pitfall is that starting the breeding season early may have the effect of reducing it at the other end, but there seems no reason why this should happen. Certainly a healthy, well-fed ferret ought to be able to stand the strain of having three litters per year.

Note added in proof June 29, 1934—Dr. T. H. Bissonnette has very kindly informed us in a private communication that he also obtained spermatogenesis in ferrets by light treatment during the 1933-4 anœstrus.

We are indebted to Major G. W. Dunkin for information as to the normal breeding season of ferrets.

VIII—*Summary*

1—Twelve female ferrets were put on to evening illumination on October 12. One came into œstrus in November, 8 in December, and 2 in January. One failed to show vulval development even after 5 months.

2—Four males were put on light at the same time. Two of these were left-castrated on November 28, after 47 days on light. Immediately afterwards they were given 10 rabbit units of urine of pregnancy extract for 6 days and then killed. The left testes contained no later stages of spermatogenesis than spermatids, but the right testes, under the influence of the U.P. extract, had just begun to produce spermatozoa, of which a few were found in the epididymis. A third light-treated male was given 10 units U.P. extract from December 18–27, at the end of which time it was fully functional and was producing masses of spermatozoa. On December 29 and January 1 it mated with light-treated females. The fourth light-treated male was killed on January 1 after 81 days light, and was found to be producing large numbers of spermatozoa.

3—It is concluded that prolonged light-treatment will activate the quiescent male ferret, but that the later stages of the process can be accelerated by administration of U.P. extract. The latter by itself has no stimulating effect on the quiescent testis.

4—The two females mated with the fertile male on December 29 and January 1 had normal pregnancies and litters of 9 and 10 respectively on February 8 and 11.

5—Experiments on pseudopregnant light-treated females showed that the post-ovulation changes take place normally even if the evening light is discontinued after ovulation.

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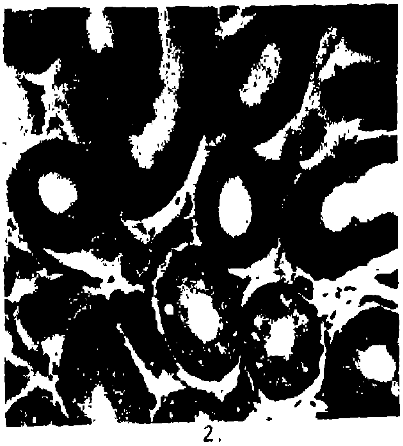
DESCRIPTION OF PLATES

Guide Letters—*SPG*, spermatogonium. *SPC*, spermatocyte. *SPT*, spermatid. *ESP*, elongating spermatid. *SPZ*, spermatozoa. *IC*, interstitial cell.

PLATE 17.

FIG. 2—Testis of FPL 17 obtained December 30 after receiving 10 units U.P. extract per day for 10 days. Testis very atrophic. × 144.



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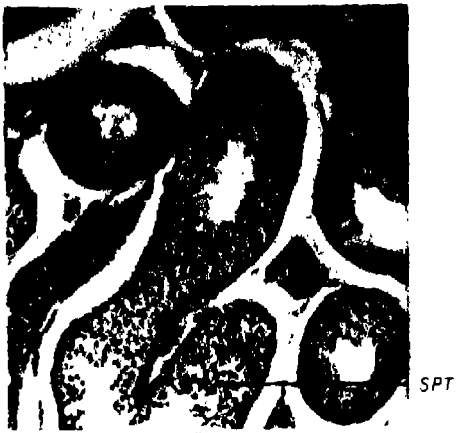

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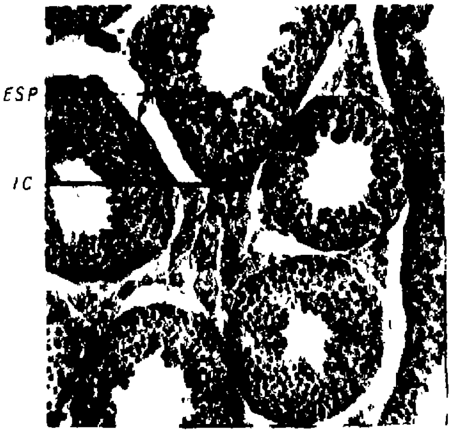

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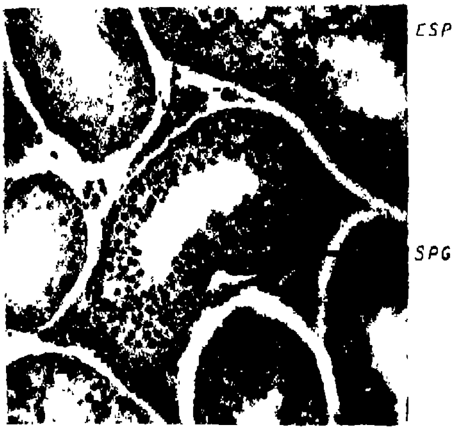

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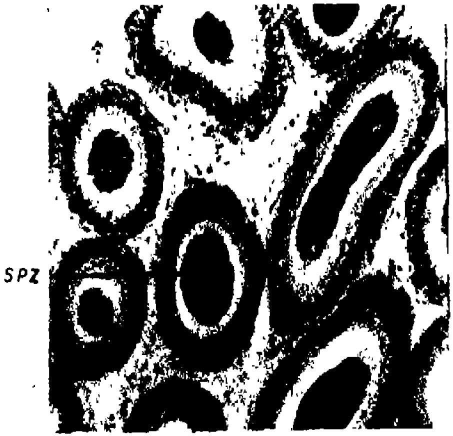

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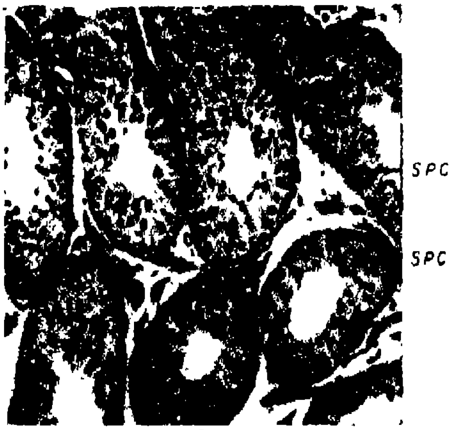

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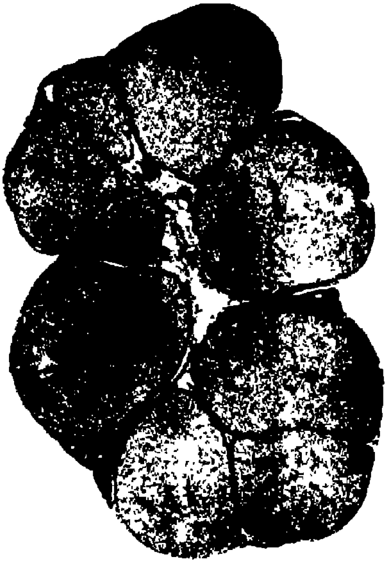

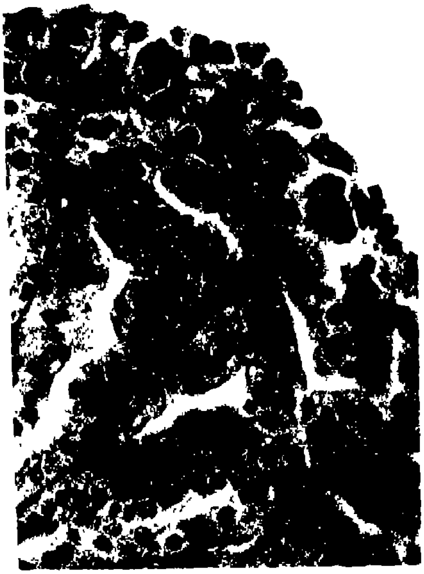

Fig. 8

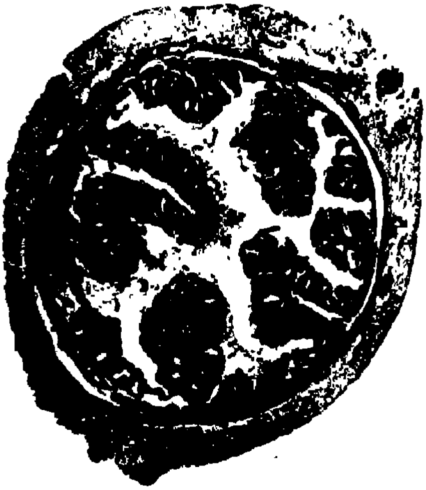

Fig 9.

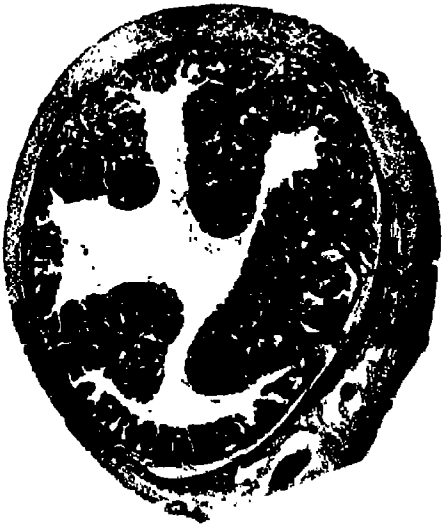

Fig 10.

Fig. 3—Testis of FPL 15, obtained on November 28 after 47 days evening light. Development to spermatid stage. × 144.

Fig. 4—Testis of FPL 13, obtained January 1 after 81 days evening light. Active production of spermatozoa. × 144.

Fig. 5—Right testis of FPL 14, obtained on December 4 after 47 days evening light, and 6 days evening light plus 10 units per day U.P. extract. Development to immature spermatozoa stage. × 144.

Fig. 6—Right epididymis of FPL 16, after 67 days evening light plus 10 days evening light and administration of U.P. extract. Masses of spermatozoa present. × 144.

Fig. 7—Testis of FPL 30, obtained on January 12, normal untreated animal. Development to secondary spermatocyte stage only. × 144.

Plate 18

Fig. 8—Ovary of FPL 11, 28 days p.c., no extra light p.c. Corpora lutea very numerous and fully developed. × 12.

Fig. 9—Uterus of FPL 5, 27 days p.c., evening illumination p.c. Normal pseudopregnant condition. × 13.

Fig. 10—Uterus of FPL 11, 28 days p.c., no extra light p.c. Normal pseudopregnant condition. × 13.

Fig. 11—Higher magnification of part of endometrium shown in fig. 9. × 200.

597 . 58 . Pseudapocryptes

The Habits and Structure of Pseudapocryptes lanceolatus, *a fish in the First Stages of Structural Adaptation to Aerial Respiration*

By B. K. Das, D.Sc., Professor of Biology in the Osmania University, Hyderabad, India

(Revised and abridged by E. W. MacBride, F.R.S.—Received April 19, 1934)

[Plates 19 and 20]

Introduction

Two short accounts of the habits of *Pseudapocryptes lanceolatus* were communicated by me to the Indian Science Congress and were published in the Proceedings of the Congress, Das (1930), Das (1932).

There are 11 Indian genera of the family Gobiidæ which include 89 species. The genus *Pseudapocryptes* has been reported from the coasts of India, Burma, the Andaman Islands, and the Malay archipelago. The specimens of *Pseudapocryptes lanceolatus* studied were obtained from the estuary of the Ganges, chiefly from Port Canning and Diamond Harbour, not far from Calcutta. The fish is often brought to the Calcutta market and is especially abundant during the months of October and November.

Structure

The fish, fig. 1, Plate 19, is eel-like, cylindrical, about 15·2 cm in length and 5·6 cm in girth. It is covered with small cycloid scales (1·1 mm × 0·7 mm) and it is very slippery because many of the ectoderm cells are glandular. It burrows in the mud in search of its prey, but when the pools in which it lives dry up, it buries itself in the wet mud beneath the superficial dried-up crust and thus avoids desiccation.

The pectoral fins are well developed; the pelvic fins are thoracic in position and are united to form a sucking disc in the manner typical of the Gobiidæ, fig. 1, B, Plate 19, and fig, 2, A, *vd*. As usual there are on each side four gill arches, each bearing two series of gill-filaments; the fourth gill is the shortest. In the first gill the outer row of gill filaments is only two-thirds as long as the inner row. Each primary filament is subdivided into a number of small leaf-like secondary filaments. Such a subdivision of the filaments has been observed by me in several species of Siluroid and Cyprinoid fish, but not so developed as in *Pseudapocryptes*. In *Gobius giuris* a typical water-breathing Gobiid, secondary subdivision of the gill filaments also occurs, but it is not strongly developed, and about one-fifth of the distal portion of each filament remains undivided. The opening of the opercular cavity of this fish is quite large and it lives for some time after being removed from water. In a small mullet (*Mugil cascasia*) which I have studied, the subdivision of the gill filaments also occurs. This fish habitually lives in clear water, but it occasionally makes excursions into muddy localities. It does not survive, however, for more than 5 or 10 minutes after being taken out of water.

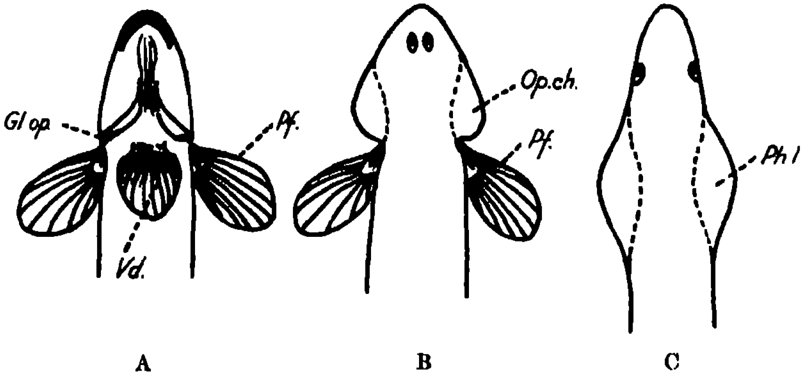

Fig. 2—A ventral, B dorsal views of the front part of *Pseudapocryptes*, ¾ size. C (for comparison) dorsal view of the front part of *Amphipnous*, ½ size. *Gl. op.*, glandular rim of operculum. *Op. ch.*, opercular chamber. *P.f.*, pectoral fin. *Ph.l.*, pharyngeal lung of *Amphipnous*. *Vd.*, ventral disc.

The opercular opening of *Pseudapocryptes*, fig. 2, A, *gl. op.*, is a narrow vertical slit about 8 mm in length, situated immediately in front of the base of the pectoral fin. The opercular chamber is sac-like, almost globular, and the opercular bones are thin and elastic; the branchiostegal membrane is supported by four elastic rays. The edge of membrane (*i.e.*, the rim of the opening) is thick and fleshy and it is covered by a thick epithelium including a large number of mucous cells. The secretion of these cells glues the opercular rim closely to the side of the body, when the fish protrudes its head from the water and begins to breathe air. The opercular chamber is thus converted into a lung; the proper air-bladder of this species, as of all Gobiidæ, is physoclistous. Parasitic copepods are very abundant, adhering to the membrane of the buccal cavity, the gill arches and the gill filaments.

The inner wall of the opercular chamber is richly vascular and is innervated by the hyomandibular branch of the 7th nerve. The lining of the buccal cavity is also richly vascular. In two groups of air breathing fish which I have described (Das, 1927), the Ophiocephalidæ and the Symbranchidæ (*Amphipnous*), this vascularity of the opercular and buccal chambers is also to be seen, but in the other air-breathing types described by me, *i.e.*, *Anabas*, *Clarias*, and *Saccobranchus*, the rich vascularity is confined to the special air-breathing organs found in these fish. On the other hand, in some small siluroid fish (*cf. Macrones cavasius* and *M. vittatus*) which are denizens of muddy water, a slight increase in the vascularity of buccal and opercular cavities can be observed. As these fish can survive an exposure to air of several hours, quite probably this vascularity is an indication of the beginning of an air-breathing habit. [The ordinary blenny (*Blennius pholis*) which is a denizen of the rock pools on the British coast can also survive for several hours out of water. —E. W. M.]

Under normal conditions *Pseudapocryptes* comes to the surface at intervals to swallow air ; the length of these intervals varies from 1 minute to 10 minutes according to the temperature and oxygen content of the water. When confined in an aquarium with impure water the fish rise at once to the surface protruding the front half of the body, as far as the pectoral fins, into the air, and they look like large tadpoles as they jostle one another in their efforts to attain the surface. In fact, one-third of the body (2 inches) may be protruded, fig. 3, Plate 19. The opercular chambers which are kept firmly closed by the pressure of their rims against the body swell out like little balloons. The closure of the opercular cavities is made more secure by the pressure against the margins of the fleshy bases of the pectoral fins. When in this condition the fish are supported by the outspread pectoral fins and float passively at the surface of the water, with their snouts protruding, fig. 4, Plate 20. After an interval of about 2 minutes they sink and then the air is expelled chiefly through the mouth, but occasionally a few bubbles are emitted from beneath the opercular rim which is slightly raised. The air is expelled by contractions of the muscles surrounding the operculum ; the contractions cause a sharp clicking sound as the fish sinks.

When under the water the fish carries on ordinary branchial respiration, making 60 to 80 breathing movements per minute under normal conditions, fig. 5, Plate 19. They sometimes cling to the side of the receptacle in which they are placed using for this purpose the ventral sucking disc composed of the pelvic fins.

When out of water under normal conditions they can progress over land using for this purpose the spread-out pectoral fins, which are employed alternately as sculls. The tail which is pressed against the ground by its lateral movements also aids in the process. They can even leap by flicks of the tail covering as much as 2 feet at a time. As they live in the tidal reaches of streams they are frequently left high and dry by the ebb-tide, and then they progress over the wet sand and mud, so as to reach the water in time to avoid being dried up. As observations on their behaviour in aquaria show, they can and do occasionally use their pectoral fins under water as they do on land, viz., to enable them to creep over the bottom.

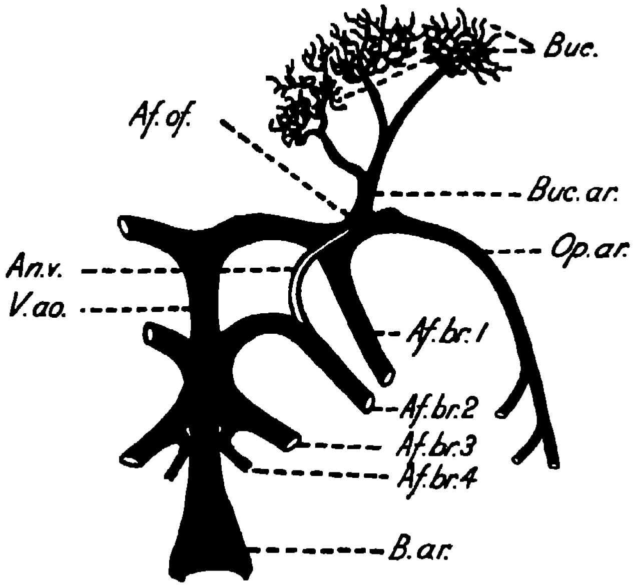

FIG. 6—The afferent branchial system of *Pseudapocryptes lanceolatus*. *Af. br.* 1, *Af. br.* 2, *Af. br.* 3, *Af. br.* 4, the first, second, third, and fourth afferent branchial arteries. *Af. of.*, afferent offshoot from branchial system (*i.e.*, bucco-opercular artery). *An. v.*, annectent vessel joining the second afferent branchial and the bucco-opercular artery. *B. Ar.*, bulbus arteriorus of the heart. *Buc. ar.*, buccal artery. *Buc. cap.*, buccal capillaries. *Op. ar.*, opercular artery.

Quite a considerable number of shore fish which exhibit no detectable modifications of structure adapted for air-breathing can live out of the water for a considerable time. Such are *Agonus* (the armed bull-head), and *Annarhicas* (the wolf fish). Among fresh-water fish the South American Siluroids *Doras* and *Callicthys* may be mentioned. *Periopthalmus*, the mud-skipper, which was formerly believed to respire through the skin of its tail, has pharyngeal pouches with a rich vascular lining similar to the pouches of *Pseudapocryptes*.

The Vascular System

As there are four gills so there are four afferent branchial arteries arising from the ventral aorta. The 3rd and 4th come off from a common stem and the 4th is much the smallest in correspondence with the reduced size of the gill which it supplies. From the first afferent vessel about a third of its length above its origin from the ventral aorta a very large artery is given off which I propose to call the *bucco-opercular artery*. It divides into two branches: one, the *buccal*, supplying the lining of the buccal cavity, and the other the *opercular*, the lining of the operculum. The opercular artery runs over the dorsal wall of the operculum and gives off six descending branches which again branch and anastomose with one another forming a network covering the inner surface of the opercular cavity. It is to be noted that whilst the bucco-opercular artery arises from the first afferent branchial artery, it also receives a small branch from the second afferent artery. The air chambers of the Ophiocephalidæ are also supplied by arteries arising from the first two afferent branchial arteries (Lele, 1932).

The blood is collected from the wall of the opercular cavity by a set of six or seven veins running upward parallel to, and alongside, the descending arteries. These veins unite in the dorsal angle of the opercular cavity to form a main *opercular* vein which enters the jugular (*i.e.*, the anterior cardinal) vein of the fish and thus reaches the heart. The anterior cardinal vein thus delivers mixed blood to the heart (as does the brachial vein in the frog which has a cutaneous feeder returning oxygenated blood from the skin). The blood which is purified in the gills is, as is normal, returned by four efferent arteries. The two first unite to form an anterior suprabranchial and the two hinder ones form a posterior suprabranchial, and these two suprabranchials on each side unite to form the dorsal aorta.

The jugular (anterior cardinal vein) also carries mixed blood in the air-breathing fish *Ophiocephalus Anabas* and *Amphipnous*.

Physiological Experiments

From a large number of experiments conducted during the months of April, May, and June, a few typical examples may be selected and their results briefly given.

(*a*) Experiment designed to test the necessity of access to air under normal conditions.

Fishes kept in ordinary tank water as already noted come to the surface at intervals of from 2 to 10 minutes in order to obtain air. Whilst under water their branchial respiratory movements average from 60 to 80 per minute. When, however, such a fish is placed in a jar of tank water at the ordinary temperature and is prevented from reaching the surface by the interposition

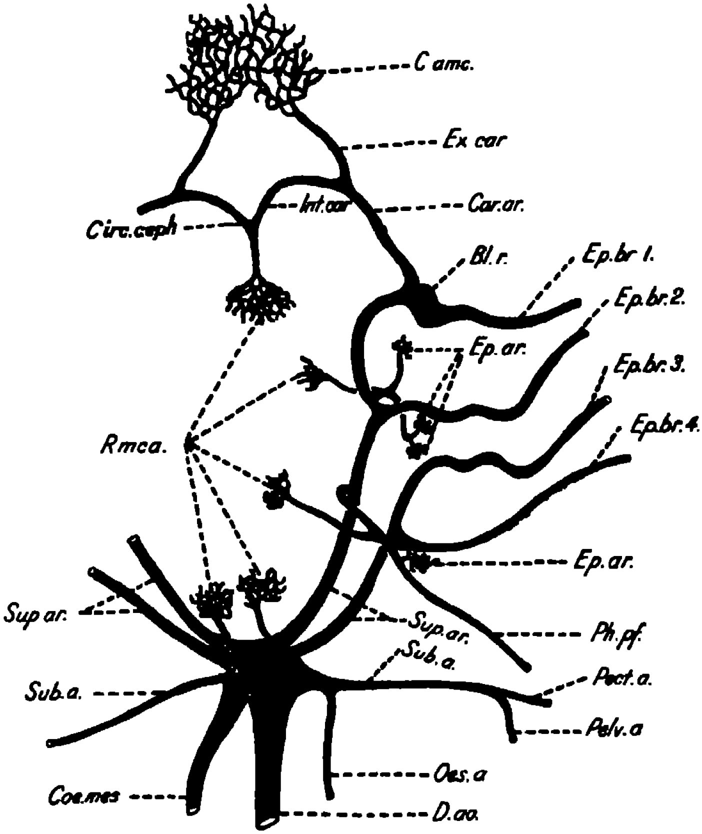

FIG. 7—Efferent branchial system of *Pseudapocryptes lanceolatus*. *Bl. r.*, small blood reservoir. *C. am. c.*, capillaries on the anterior part of the buccal cavity. *Car. ar.*, carotid artery. *Circ. ceph.*, circulus cephalicus. *Col. mes.*, coeliaco-mesenteric artery. *D. ao.*, dorsal aorta. *Ep. ar.*, small arteries supplying the epibranchial regions of the 1st, 2nd, and 3rd gill arches. *Ep. ar.*, similar artery supplying the epibranchial region of 4th arch. *Ep. br.* 1, *Ep. br.* 2, *Ep. br.* 3, *Ep. br.* 4, the first, second, third, and fourth epibranchial arteries. *Ex. car.*, external carotid artery. *Int. car.*, internal carotid artery. *Oes. a.*, œsophageal artery. *Pect. a.*, artery to pectoral gill. *Pelv. a.*, artery to pelvic fin. *Ph. pf.*, artery to the posterior part of the floor of the pharynx. *R. mca.*, small arteries to the posterior part of the roof of the pharynx. *Sub. a.*, subclavian artery. *Sup. ar.*, suprapharyngeal arteries.

of a perforated zinc plate sunk a few inches in the water, then it at first rises about every 5 minutes and dashes its snout against the plate in the endeavour to reach the air. When it has failed to do so a number of times, it becomes much excited, dashing to the surface at least once a minute; meanwhile branchial respiration under the water becomes much accelerated, the movements sometimes reaching 150 per minute. Complete asphyxiation and death transpired in 15 to 20 hours. It will be recollected (Das, 1927) that the complete asphyxiation of *Anabas* under similar circumstances was accomplished in 35–40 minutes; and therefore the need of *Anabas* for aerial respiration is much greater than that of *Pseudapocryptes*.

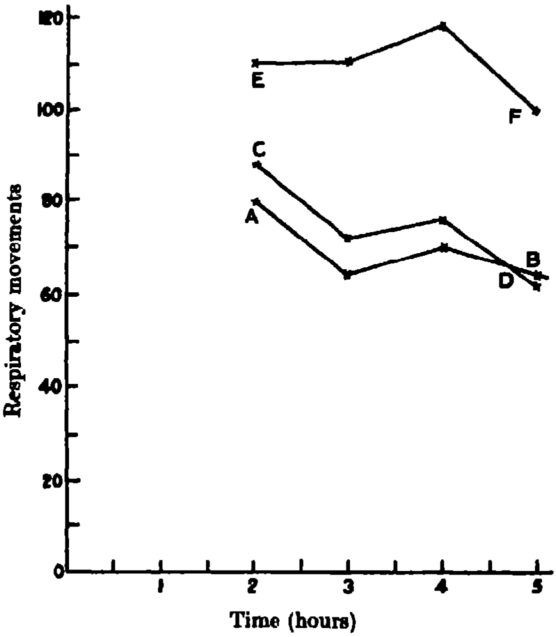

Fig. 9—The graphs showing change in branchial respiratory movements with time, in three specimens of *Pseudapocryptes* during asphyxiation by exclusion from access to air.

(*b*) Experiments to show the necessity of intact opercular chambers for air breathing.

When specimens of *Pseudapocryptes* are removed from the water entirely and left on the marble floor of the tank room they die from desiccation in times varying from 40 minutes to 2 hours. But until they become completely exhausted they continue rapid air breathing. The opercular chambers may be seen to swell out and collapse at intervals varying from 5 seconds to 2 minutes.

When one operculum is removed the fish endeavours still to swallow air and the intact opercular chamber carries out respiratory pulsations. These pulsations ceased in 50–60 minutes and all trace of body movement stopped, death ensuing in one fish in 1 hour and 8 minutes; in another fish in 1 hour and 40 minutes.

When both opercula are removed the fish continues gasping movements and air escapes through the gill slits. Death ensued in 1 hour and 30 minutes.

A control fish with uninjured opercula lived for 3 hours.

In conclusion, I wish to express my thanks to my former teacher, Professor E. W. MacBride, for revising and condensing the original MSS of this paper and communicating it to the Society for publication. I have to thank also my friend Mr. R. Norman, Keeper of Fish in the British Museum, for checking the specific names used in the paper. My thanks are also due to my friends Messrs. T. N. Podder, B.Sc., G. K. Chakravorty, M.Sc., H. P. Roy, M.Sc., and G. M. Das, M.Sc., for help in carrying out the physiological experiments.

Summary and Conclusions

The facts adduced in this paper justify the conclusion that aerial respiration at intervals depending on the temperature is a physiological necessity to *Pseudapocryptes lanceolatus* in the conditions under which it lives. It is also clear that the air is absorbed mainly by the lining of the opercular chamber and that this chamber is adapted to this function by a rich vascular supply and by the thinness and elasticity of the opercular bones. Further, there is an adaptation which enables the operculum temporarily to be completely closed. It has long been known that fish belonging to the families Cyprinoidea and Siluroidea which show no structural adaptations to air breathing will nevertheless come to the surface and swallow air when the oxygen tension in the waters in which they live is low. [In England the stickleback *Gasterosteus* has been observed leading a healthy life in water so polluted with sewage that it contained no oxygen whatever. Under these circumstances the fish must have depended entirely on aerial respiration.—E. W. M.]

This casual resort to air has been termed "Notatmung." *Pseudapocryptes* therefore stands midway between fish such as these and more specialized air breathers like *Anabas* and *Clarias* where definite respiratory organs are developed as outgrowths from the gill-arches and project into the opercular cavity. In these the rich vascular supply is confined to the specialized organs. The *Ophiocephalidæ* are in an intermediate condition between *Pseudapocryptes* and *Anabas*. In them the inhaled air is received into dorsal pockets of the pharynx. But not only these pockets but also the whole dorsal wall of the pharynx has a rich vascular supply and doubtless respiratory activity is shared by the whole dorsal wall of the pharynx.

The study of air breathing fishes gives the clearest proof that in evolution, function changes first and modified structure follows long after, when changed function has lasted long and become deeply ingrained in the constitution.

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DESCRIPTION OF PLATES 19 and 20

Fig. 1—A, lateral; B, ventral; C, dorsal views of *Pseudapocryptes lanceolatus*. About two-thirds natural size. *Gl. op.*, glandular opercular rim. *Vd.*, ventral (*i.e.*, pelvic) fins.

Fig. 3—Behaviour of a group of *Pseudapocryptes* kept in a bucket with foul water. The fish are all rising to the surface and inhaling air. Two of them by the aid of their ventral discs and pectoral fins have succeeded in protruding their bodies 2 inches from the water.

Fig. 4—Behaviour of *Pseudapocryptes* after having inhaled air (about one-third natural size). The fish are seen with swollen opercular chambers hanging vertically from the surface of the water.

Fig. 5—A number of *Pseudapocryptes* resting on the bottom of the tank after having exhaled the air which they had swallowed.

Fig. 8—Inner aspect of the right operculum of *Pseudapocryptes lanceolatus* showing the vascular supply (Bloch and Schneider). *Op.*, attachment of operculum to skull, cut across. *Op. v.*, opercular vein. *Op. a.*, opercular artery. *Op. r.*, opercular fleshy rim.

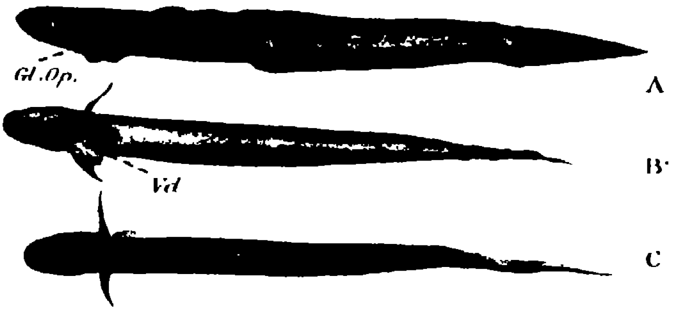

Fig. 1

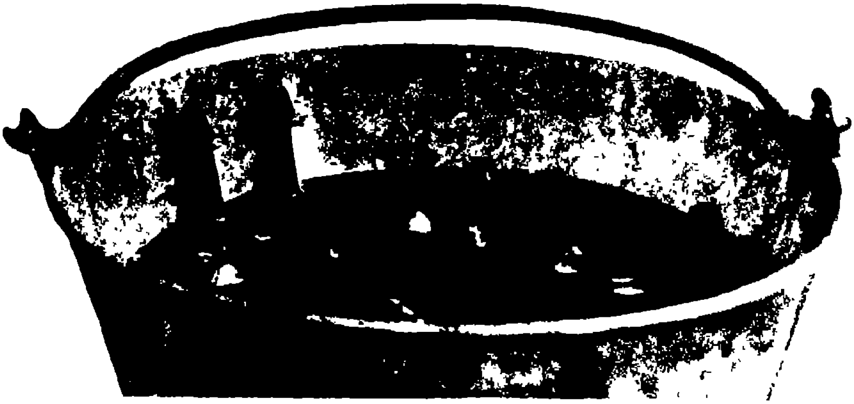

Fig. 3.

Fig. 5.

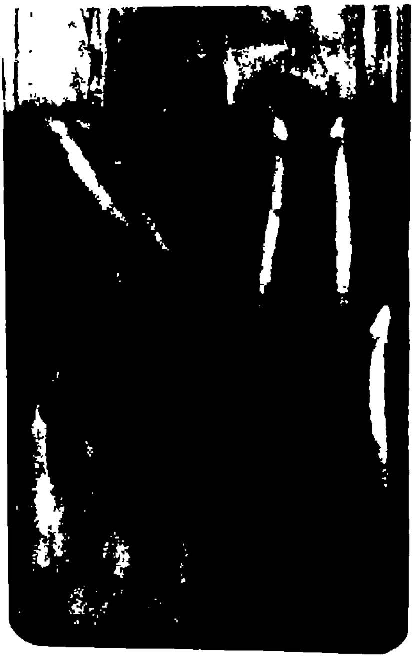

FIG 4

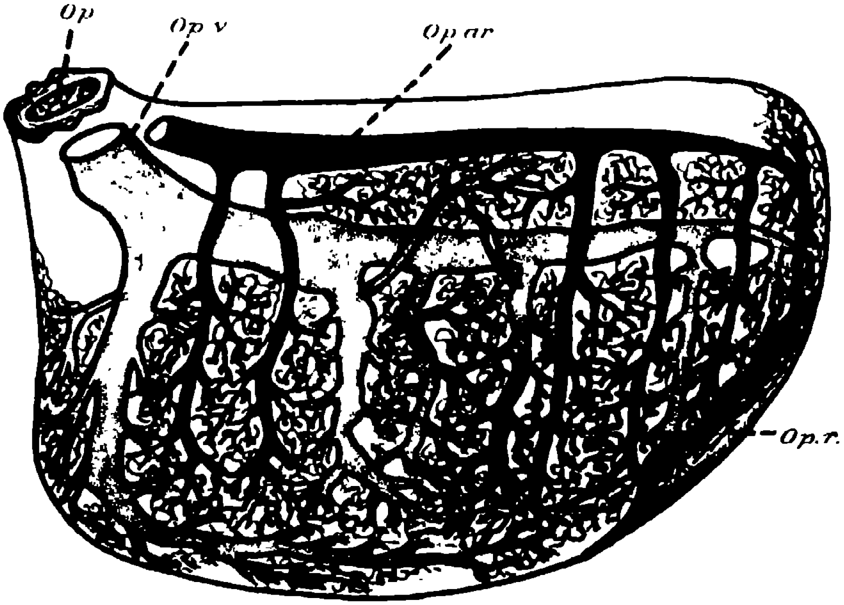

FIG. 8.

616 . 89 – 008 . 454 . 65

The Relative Aetiological Importance of Birth Order and Maternal Age in Mongolism

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I

Mongolian imbeciles are very often born last in a long family. This fact, which was pointed out many years ago by Shuttleworth (1909), has led clinicians to believe that mongolism is to some extent a product of the exhaustion of maternal reproductive powers due to frequent child-bearing (Still, 1927; Fantham, 1925). The conclusion is widely accepted with the reservation that the affected child is not necessarily born at the end of the family (Thompson, 1925). Several cases are first-born, in fact, and it is sometimes stated that the *condition occurs more frequently in first and last children than in other ordinal* positions. There is, however, ample evidence that mongolian imbeciles have a significantly later birth rank than normal children (Hogben, 1931).

It is also established, from large numbers of figures which have been collected, that the maternal age at the birth of mongolian imbeciles is unduly high. Though some of these imbeciles have young mothers, most of the cases (about 70%) are born after the mother has reached the age of 35 years. Thus the maternal age itself is likely to be an aetiological factor quite as important as birth order. I know of no serious attempt, however, to distinguish between the aetiological significance of these two factors: to do this is the task I have undertaken.

In an article published recently (Penrose, 1933) I have attempted to show how statistical methods may be used to disentangle the probable aetiological effects of paternal and maternal age in mongolism. The results indicated clearly that paternal age need not be considered a significant causal factor; we shall therefore not refer to it in the present discussion.

There is another factor which has been asserted by some writers to be of causal significance. The interval between the birth of a mongolian imbecile and the birth of the child which immediately precedes it is found, on the average, to be significantly longer than the corresponding interval preceding normal births (van der Scheer, 1927); that is, the mongol apparently follows a period of diminished fecundity (Jenkins, 1933). This question of length of preceding interval is intimately bound up with the main question I am considering here and it will be referred to in the course of the discussion.

The investigation of the facts, on which the present argument is based, was carried out as part of the work of the research department of the Royal Eastern Counties' Institution. It involved the accurate determination of the maternal age at the birth of all offspring in 217 sibships each containing one or more mongolian imbeciles. The birth order was also recorded with particular care: miscarriages and stillbirths were deemed to affect the ordinal number of subsequent births, but they have been excluded from the data as presented here. It is very uncertain whether they represented offspring affected or not with mongolism and I wished to include in the data only those individuals in the 217 sibships of whom it could be said with certainty that they were either mongolian imbeciles or normal. The sibships, giving the birth rank and maternal age for each known individual, are set out at the end of the paper, and a summary is given in Table I. Altogether these particulars concerning 1031 persons, 807 normal children and 224 mongolian imbeciles, are recorded. A glance at the distribution is sufficient to show that there is good reason for supposing that both the birth ranks and the maternal ages are significantly higher for affected than for normal children. About 60% of the affected children are born last and the great majority of the families are completed. We will proceed to investigate how far this displacement of affected offspring towards the end of the sibship can be attributed to the late maternal ages at which affected offspring are most frequently born.

II

The following constants were calculated from the distribution given in Table I.

	Number	Mean maternal age at birth ($\bar{q}$)	Standard deviation (σ_q)
		years	years
Affected children (M) .	224	37·415	—
Normal children (N)	807	31·312	—
Total children (T)	1031	32·638	6·778

The mothers are, on the average, more than 6 years older at the births of the affected than at the births of the normal individuals. This is a highly

Table I—Scatter Diagram showing Relationship of Maternal Age to Birth Rank

(Suffixes in bold type indicate Mongols)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total	(N)	(M)
17	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	—
18	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	3	—
19	8_3	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	9	6	3
20	11_1	3	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	15	14	1
21	12_3	7	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	20	17	3
22	7_2	9_2	3	1	—	—	—	—	—	—	—	—	—	—	—	—	—	20	16	4
23	13_2	11_2	5	1	—	—	—	—	—	—	—	—	—	—	—	—	—	30	26	4
24	16_1	10	13	4	—	—	—	—	—	—	—	—	—	—	—	—	—	43	42	1
25	15	13	6	2	1	2	—	—	—	—	—	—	—	—	—	—	—	39	39	—
26	9_2	13	10_1	9	3	—	—	—	—	—	—	—	—	—	—	—	—	44	41	3
27	5_1	13_3	9_1	8_1	10	—	1	—	—	—	—	—	—	—	—	—	—	46	40	6
28	11_1	9_1	5	3	6	4_1	2	1	—	—	—	—	—	—	—	—	—	41	38	3
29	6_1	7_1	9	10	4	5	1	1	—	—	—	—	—	—	—	—	—	43	41	2
30	8	10_1	11_1	6	6	5	5	—	2	—	—	—	—	—	—	—	—	53	51	2
31	5_1	4	9_1	5_1	9	8_1	2	2	1	1	—	—	—	—	—	—	—	46	42	4
32	5_1	13_2	4_1	7	8	7_1	6	2	2_1	—	—	—	—	—	—	—	—	54	48	6
33	5	5_3	7_2	8	7_1	8	6	2	2	1	—	—	—	—	—	—	—	51	45	6
34	3	9_2	8_4	5_2	9	2	6	6	1_1	1	1	—	—	—	—	—	—	51	42	9
35	3_1	2_1	8_1	7_1	8_2	10_3	10_2	8_2	3	2	—	1	—	—	—	—	—	62	49	13
36	2_1	3_3	4	5_2	5	6	1	6_1	3	4	3_1	—	—	—	—	—	—	42	34	8
37	1	2	4_3	8_4	4_1	4_1	3_2	7_3	5_1	3	1	—	1	—	—	—	—	43	28	15
38	2_2	4	1	4_2	5	5_2	11_6	1	4_1	3_1	2	2	1	1	—	—	—	46	32	14
39	2_1	1_1	4_2	7_3	5_1	3_1	5_2	2	3_1	4_2	1	3	1	—	—	—	—	41	27	14
40	1_1	3_1	4_1	2_1	2_1	6_3	9_4	2_2	4_2	—	2	2	1_1	1_1	—	—	—	39	21	18
41	—	1	5_2	3_1	—	—	3_2	4_2	2_1	6_3	4_2	4_2	1_1	1	—	—	—	34	18	16
42	—	3_3	5_5	3_3	2_1	2_2	2_1	5_3	1	2_1	2_1	2	4_2	1	1_1	—	—	35	12	23
43	—	1_1	1_1	2_1	2_2	3_2	—	4_2	2_1	1	—	1	1	1	3_2	1	—	23	11	12
44	—	—	—	—	4_3	4_4	2	3_2	2_1	—	—	—	2_1	2_1	—	1	—	20	8	12
45	1_1	—	—	—	—	2_1	1_1	1	4_1	1_1	1_1	1	1	1	—	—	1_1	15	8	7
46	—	—	2_2	1_1	1_1	1_1	—	1	1_1	1_1	—	—	2	2_1	1	—	1	14	6	8
47	—	—	—	—	—	—	—	2_2	1_1	1	—	—	1_1	—	1_1	—	1_1	7	1	6
48	—	—	—	—	—	—	—	—	—	—	—	—	—	1_1	—	—	—	1	—	1
Total .	154	157	139	112	101	87	76	60	43	31	17	16	16	11	6	2	3	1031	—	—
(N) .	128	130	111	89	88	64	56	41	30	22	12	14	10	7	2	2	1	—	807	—
(M) . . .	26	27	28	23	13	23	20	19	13	9	5	2	6	4	4	—	2	—	—	224

significant difference, for it is more than 10 times the standard error, which is $\sigma_a . \sqrt{T/MN}$ or 0·51 years.

The following results are obtained, by making similar calculations, for birth order.

	Number	Mean birth rank* ($\overline{f}$)	Standard deviation (σ_f)
Affected children (M)	224	5·65	—
Normal children (N) . .	807	4·61	—
Total children (T)	1031	4·83	3·34

* The concept of mean birth rank (mean ordinal position) is a statistical fiction useful in this problem; it involves using imaginary units—fractions of a place or rank—which have no meaning except in averages. The averages given here are therefore simply shorthand description of complicated distributions.

The difference between the mean birth ranks of affected and normal children is 1·04 with a standard error ($\sigma_f . \sqrt{T/MN}$) amounting to 0·25. There is a significant discrepancy, but it is not so marked as in the case of maternal age.

In considering the question of birth order, we have not yet allowed for the effect brought about by the presence in the data of families of varying sizes. If human families were all of uniform size the expected mean birth rank, in any group of selected individuals distributed at random in respect of position, would be obtained by the ordinary methods of averaging. But human families vary in size from 1 to 20 or more pregnancies resulting from the same union. When families are selected, as they are here, by the presence of at least one affected member, the proportion of affected to normal children is greater in the small families than in the large. When the families in data such as these are pooled, the concentration of affected individuals in short sibships with low birth ranks causes the mean birth rank of affected children to be relatively nearer to the beginning of the family than it would be if we were dealing with a representative sample of the general population. Neglecting to take this consideration into account has led Pearson (1907), for example, to assert that the first-born are more likely than children occupying the later birth ranks to be affected by certain diseases, e.g., tuberculosis. Yule and Greenwood (1914) have criticized Pearson's conclusion and it is now generally accepted that, in estimating the probable number of affected children in each birth rank, the varying sizes of the families must be taken into consideration and accordingly weighted in the inverse ratio of their sizes. If more than one individual is affected in the same sibship, it is necessary to count the sibship once over for each affected member; that is to say, the sibships must be weighted directly according to the number of affected individuals they contain.

The simple method, sometimes known as the Yule-Greenwood reconstruction (Thurstone and Jenkins, 1931), by means of which a good approximation is obtained to the expected number of affected children in each birth rank (Hogben, 1931) is not suitable to the present data owing to the gaps left for siblings of unknown type. It is necessary to modify the method so that we may treat each sibship separately.

Let s be the number of known individuals recorded in the sibship and let a be the number of affected individuals in this sibship. The expected number of affected individuals in each birth rank, if they are distributed at random, is a/s. In each sibship only those ordinal positions already occupied are to have expectations attributed to them. In this way a table of expectations has been made out whose precision is equivalent to the accuracy of the original data. Examples of the expectations attributed by this method to various ordinal positions in different sibships are shown below:—

Serial number of sibship	Order of birth 1st	2nd	3rd	4th	5th	6th	7th
123	0·200	0·200	0·200	0·200	—	0·200	—
4	—	—	—	—	—	—	1·000
102	0·167	—	0·167	0·167	0·167	0·333	—
198	—	—	0·500	0·500	0·500	0·500	—

Sibship No. 123 shows how gaps are left for unknown individuals (miscarriages, etc.).

In sibship No. 4 the mother had other children by different fathers; the half-sibs are purposely not recorded here.

In sibship No. 102 twins occur; two individuals occupy the same birth rank (the 6th) and the expectation at this place is consequently doubled.

In sibship No. 198 there are two affected persons, hence the total expectation is 2; the sibship is, in fact, counted twice over, once for each affected member.

The sum of the columns of expectation in each ordinal place gave, for the whole 217 families, the values shown in Table II; they are compared with the observed numbers of mongols.

These expectations, Table II, are the probable numbers of affected individuals in the 217 sibships, if the 224 affected children were distributed at random in respect of birth rank.

The mean ordinal place in family ($\bar{e}_M$) of affected individuals, if thus distributed at random, is now estimated from the expected numbers by the usual process of averaging. Thus,

$$\bar{e}_M = \frac{(1 \times 44\cdot 99) + (2 \times 43\cdot 40) + (3 \times 33\cdot 09) + \ldots}{224}$$

$$= 4\cdot 10 \text{ ordinal places.}$$

Table II

Rank	Expected number affected (e_M)	Oberved number affected (f_M)
1st	44·99	26
2nd	43·40	27
3rd	33·09	28
4th	24·46	23
5th	18·63	13
6th	15·15	23
7th	13·32	20
8th	9·65	19
9th	6·47	13
10th	4·91	9
11th	2·10	5
12th	2·45	2
13th	2·56	6
14th	1·60	4
15th	0·76	4
16th	0·17	0
17th	0·30	2

The expected number of normal individuals in each birth rank is obtained by subtracting the numerical values given above from the totals (t) in each place, thus:—

Order of birth	t		e_M		e_N
1st	154	−	44·99	=	109·01
2nd	157	−	43·40	=	113·60
etc.					

The corresponding mean value for expected positions of normals ($\bar{e}_N$) is found to be 5·04.

The differences between the observed mean values ($\bar{f}_M$ and $\bar{f}_N$) and these expected mean values ($\bar{e}_M$ and $\bar{e}_N$) indicate the real average displacements of the mongolian imbeciles and normals. The displacements $\bar{f}_M - \bar{e}_M$ and $\bar{f}_N - \bar{e}_N$ are greater than the corresponding differences of $\bar{f}_M$ and $\bar{f}_N$ from the mean value $\bar{f}$ which have already been given above. Incidentally, if all the sibships had been of the same size, each containing the same number of affected children, $\bar{e}_M$ and $\bar{e}_N$ would have both been equal to $\bar{f}$. In the present instance the total difference between the mean birth ranks of affected and normal children is really greater than it at first sight appeared to be; it is increased by the reconstruction to 1·98, *i.e.*, $(\bar{f}_M - \bar{f}_N) - (\bar{e}_M - \bar{e}_N)$. This displacement is now more than seven times the standard error, 0·25. The effect of birth rank on the incidence of mongolism is therefore apparently of great significance, though it is not quite as marked as the maternal age effect.

III

We are now in a position to calculate the expected number of affected children occupying each birth rank on the assumption that we only know the maternal ages for these individuals and not the orders of their births. It is necessary first to calculate for any given rank the size of the deviation from M/T (the average proportion affected) which can be attributed to the effect of the maternal ages corresponding to the affected children in the given rank.

We turn our attention to the first born and we find, from Table I, that the mean maternal age for first-born children (q_1) is 26·071 years.

In order to find the most probable *proportion* of affected to total first-born children, based on the knowledge of this mean maternal age, we use a regression which can be obtained from the distribution of affected and normal (M and N) given in the upright columns on the extreme right in Table I. The most probable deviation at any given maternal age, q, from (M/T) or 224/1031, the mean proportion affected, is given by multiplying $q - \bar{q}$ by b, where

$$b = r_{mq} \cdot \frac{\sigma_m}{\sigma_q}.$$

The correlation r_{mq} is a simple biserial,

$$r_{mq} = \frac{(\bar{q}_M - \bar{q}_N) \cdot m}{q} \quad \text{and} \quad \sigma_m = \sqrt{\mathrm{MN}}/\mathrm{T}.$$

Substituting the values which are already known, we find that $b = 0{\cdot}0226$.

Since $q_1 = 26{\cdot}071$,

$$\begin{aligned} b\,(q_1 - \bar{q}) &= (0{\cdot}0226)\,(26{\cdot}071 - 32{\cdot}638) \\ &= -\,0{\cdot}1483. \end{aligned}$$

From this fraction we obtain the expected numerical deviation in first born affected individuals by multiplying it by the total number of first-born children, 154.

Thus, $$(-\,0{\cdot}1483)\,(154) = -\,22{\cdot}84.$$

The standard error of this expected deviation is

$$\sigma_m \cdot \sqrt{1 - r_{mq}^2} \cdot \sqrt{154}, \text{ i.e., } \pm\, 4{\cdot}75.$$

Returning now to the figures given when the results of the reconstruction were compared with the observed numbers of mongols in each birth rank, § II, we ascertain the following facts :—

Total first-born children (t_1)	154
Observed number affected (f_{M1})	26
Number of first born estimated by reconstruction of the data on the assumption that affected children are distributed at random with respect to birth order (e_{M1})	44·99

Since the families in the data are not uniform in size, the average values, $t_1 . \mathrm{M/T}$, $t_2 . \mathrm{M/T}$, etc., do not represent the expected numbers of mongols in each rank if birth order is random. The average $t_1 . \mathrm{M/T}$ has to be replaced by $e_{\mathrm{M}1}$ or 44·99. The deviation due to the effect of maternal age must be added to $e_{\mathrm{M}1}$ to obtain the expected number of first-born mongols. We therefore add − 22·84 to 44·99 and the result, 22·15, differs from the observed number, 26, by 3·85. This difference is less than the standard error, 4·75, and is insignificant.

Table III—Results of Analysis by First Method

I	II	III	IV	V	VI	VII	VIII
1	154	26	44·99	−22·84	22·15	+3·85	4·75
2	157	27	43·40	−14·80	28·60	−1·60	4·80
3	139	28	33·00	−5·28	27·81	+0·19	4·52
4	112	23	24·46	−0·46	24·00	−1·00	4·05
5	101	13	18·63	+1·75	20·38	−7·38	3·85
6	87	23	15·15	+4·69	19·84	+3·16	3·57
7	76	20	13·32	+5·59	18·91	+1·09	3·34
8	60	19	9·65	+6·97	16·62	+2·38	2·97
9	43	13	6·47	+5·64	12·11	+0·89	2·51
10	31	9	4·91	+4·34	9·25	−0·25	2·13
11	17	5	2·10	+2·53	4·63	+0·37	1·58
12	16	2	2·45	+2·75	5·20	−3·20	1·53
13	16	6	2·56	+3·52	6·08	−0·08	1·53
14	11	4	1·60	+2·66	4·26	−0·26	1·27
15	6	4	0·76	+1·54	2·30	+1·70	0·94
16	2	0	0·17	+0·49	0·66	−0·66	0·54
17	3	2	0·30	+0·91	1·21	+0·79	0·66
Mean birth rank (or corresponding deviation from mean birth rank)	4·83	5·05	4·10	+1·55	5·65	0·00	0·17

I. Birth rank.
II. Total children in each rank, t_1, t_2, t_3, etc.
III. Observed number of mongols, $f_{\mathrm{M}1}$, etc.
IV. Expected number of mongols, based upon reconstruction, $e_{\mathrm{M}1}$, etc.
V. Expected deviation in number affected, based on mean maternal age, in a given birth rank, $b\,(q_1 - \bar{q})\,t_1$, etc.
VI. Expected number of mongols, $e_{\mathrm{M}1} + b\,(q_1 - \bar{q})\,t_1$, etc.
VII. Difference between observed and expected numbers, $f_{\mathrm{M}1} - e_{\mathrm{M}1} - b\,(q_1 - \bar{q})\,t_1$, etc.
VIII. Standard error of expected numbers, $\sqrt{\mathrm{MN/T}} . \sqrt{1 - r_{mq}^2} . \sqrt{t_1}$, etc.

The same calculation and comparison has been made for other birth ranks. The figures are shown in Table III. The fit between observed and expected values in columns III and VI is satisfactory. There is no indication that any birth rank is more frequently occupied by a mongol than would be expected from the mere consideration of the maternal ages at which these children are born. Although the observed number of first-born mongols is slightly in excess of expectation, the excess is not significant. Primogeniture is therefore not likely to be an aetiological factor.

IV

There are certain inaccuracies in the statistical treatment I have so far employed here. It has been assumed that the reconstruction is entirely independent of maternal age. This is, however, not necessarily true, for the smaller families might be concentrated more at the low or high maternal ages. In section III it was tacitly assumed that the regression of incidence of mongolism on maternal age is adequately represented by a straight line: this assumption is inaccurate. Moreover, no allowance has been made for the possible effect of the reconstruction upon the sampling errors given in Table III. To avoid these sources of ambiguity the data have been subjected to analysis by an entirely different method which was suggested by Professor R. A. Fisher.* By use of this new process we are able, after a single complex reconstruction, to compare the observed number of mongols in any given birth rank with the number which is to be expected on the hypothesis that the probability of a mongol child depends upon maternal age (in some manner unknown prior to the data) but not, given age, upon birth rank.

Let us suppose that there are a number of families containing only two children born at the maternal ages of 32 and 42, respectively, and that one child in each family is a mongol. Call p_{32} and p_{42} the probabilities that a mongol is born at these maternal ages. The frequencies of families which have the mongol at age 32 to those which have the mongol at 42 will be in the ratio

$$\frac{p_{32}}{1-p_{32}} : \frac{p_{42}}{1-p_{42}},$$

or, say, $x_{32} : x_{42}$ where x is proportional to $\frac{p}{1-p}$. In any such family the expectation that the child born at 32 is a mongol is $\frac{x_{32}}{x_{32}+x_{42}}$. In general, for families containing only one mongol the expectation that any given child is the affected one is $x/S(x)$ where $S(x)$ is the sum of the values of x for the different maternal ages represented in the family. For families containing more than one mongol the corresponding expressions are more complicated. Given a series of x values, the expectation that each recorded child in the data is a mongol can be calculated. These expectations are then summed up in two ways. In the first place, the assigned values of x will be correct when the number of mongols observed at any given maternal age tallies with the sum of the expectations attributed to each child at that maternal age. Secondly, when the correct x values have been ascertained, the sum of the expectations for all the children in any given birth rank can be compared with the number of mongols actually observed in that birth rank.

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* Only a brief account of the method is given here; a full description is to appear in the 'Annals of Eugenics.'

In order to simplify the arithmetic, maternal ages for each five consecutive years were grouped together. The following x values for these groups were estimated by a method of successive approximation :—

	Maternal age group	x values
A	15 to 19	22
B	20 to 24	10
C	25 to 29	6
D	30 to 34	19
E	35 to 39	88
F	40 to 44	296
G	45 to 49	558

It will be seen that the probability of the birth of a mongol child is lowest between the maternal ages of 25 and 29 and that it rises rapidly after the age of 35. Table IV shows the results of calculating the expectation of mongolism for each child recorded in the data. The x values have been chosen so that the sum of the expectations (in the vertical columns) agrees sufficiently closely

Table IV—Results of Analysis by Second Method

Birth order	A	B	C	D	E	F	G	Calculated total	Observed total
1	2·92	7·33	4·44	2·34	4·94	1·00	1·00	23·97	26
2	0·03	4·23	4·90	6·84	4·46	6·35	—	26·80	27
3	0·03	1·12	1·78	5·84	7·94	12·11	1·94	30·76	28
4	—	0·19	1·48	3·30	11·32	6·00	1·00	23·29	23
5	—	—	0·76	3·05	6·04	6·65	0·54	17·04	13
6	—	—	0·26	2·30	7·99	8·89	2·21	21·65	23
7	—	—	0·18	1·37	8·55	8·06	0·94	19·10	20
8	—	—	0·02	0·80	4·57	9·55	2·43	17·37	19
9	—	—	—	0·63	4·07	4·83	3·25	12·78	13
10	—	—	—	0·09	2·11	5·02	1·90	9·12	9
11	—	—	—	0·02	0·83	3·24	0·42	4·51	5
12	—	—	—	—	0·85	2·55	0·55	3·95	2
13	—	—	—	—	0·37	3·42	1·86	5·65	6
14	—	—	—	—	0·11	1·86	1·57	3·54	4
15	—	—	—	—	—	1·32	0·91	2·23	4
16	—	—	—	—	—	0·61	—	0·61	0
17	—	—	—	—	—	—	1·65	1·65	2
Calculated total	2·98	12·87	13·82	26·58	64·14	81·46	22·17	224·02	—
Observed total ...	3	13	14	27	64	81	22	—	224

with the total number of mongols observed in each of the maternal age groups. The expectations, added horizontally, give the expected numbers of mongols in each birth rank. The discrepancies between observed and expected values are not great, but there is a slight excess of first-born mongols, as in the results which were obtained by the earlier method of analysis, given in Table III. In calculating the standard error of these differences it was found convenient to group the birth ranks, and the following results were obtained :—

Birth order	Observed number of mongols	Expected number of mongols	Difference	Standard error
1st	26	23·97	+2·03	2·68
2nd or 3rd	55	57·56	−2·56	3·85
4th, 5th, or 6th	59	61·98	−2·98	4·07
7th to 10th	61	58·37	+2·63	3·41
11th to 17th	23	22·14	+0·86	1·84
Total	224	224·02	—	—

The agreement between the theoretical number of mongols and the observed number is satisfactory, and the excess of first born affected children is not significant.

V

If we accept that order of birth is not a significant aetiological factor in mongolism, it becomes very doubtful whether the length of the interval preceding the birth can be considered to be of aetiological significance either. If order of birth is not causal, it makes no difference whether a mother aged 40, bearing an affected child, has previously had only one child, born 20 years earlier, or whether the gap has been filled by 10 pregnancies. The present data, however, give opportunities for testing directly the hypothesis that the interval preceding the birth of a mongolian imbecile is unduly long.

If we take only those cases which are last born and which immediately succeed a normal child in the sibship, we shall find that the interval between these two, ultimate and penultimate, births is very long in comparison with intervals between two normal children in the sibships. This is to be expected if maternal age is the significant aetiological factor, for, during an interval between births, the maternal age increases but the birth rank does not rise correspondingly. The longer the interval, therefore, the more likely will it be that an offspring is affected. If, however, instead of taking affected children who are born last, we select those who both succeed immediately and are immediately followed by a normal child, a comparison may be made of the intervals before and after the birth of affected offspring. Sibships 8, 20, 21, 30, 33, etc., contain cases fulfilling this condition. There are in all 37 instances of this in 36 sibships. The mean intervals between consecutive births are shown below.

Mean interval between normal and mongol immediately following, 2·78 ± 1·33 years.

Mean interval between mongol and normal immediately following, 2·95 ± 1·82 years.

The difference between the two means is 0·17 years with a standard error ± 0·37.

The observed difference is not significant, but it actually shows the interval succeeding the mongolian imbecile to be *greater* than that preceding it. This is probably due to the fact that, normally, as maternal age increases, intervals in the sibship tend slightly to lengthen. The large interval or period of diminished fecundity found in certain instances preceding the birth of a mongolian imbecile is, according to this analysis, unlikely to be of causal significance.

VI

In this paper data from the investigation of the family histories of 224 mongolian imbeciles are presented. Statistical analysis, by two entirely different methods, shows that the numbers of imbeciles in each birth rank are very close to those which are to be expected on the assumption that the incidence of mongolism depends upon maternal age and not upon birth order. This conclusion applies also to the number of first-born children found to be affected. Aetiological significance can therefore not be attributed to birth order with any reasonable degree of probability. Neither is there any evidence that the long interval which sometimes precedes the birth of an affected child is of causal significance.

These findings are comparable with Wright's observations concerning the relative effects of birth order and maternal age in the production of certain characteristics in the guinea pig (Wright, 1926); here, too, birth order was found to be of no significance.

I wish to thank all those who have taken part in preparing the material presented in this paper and particularly my assistants, Miss D. E. Newlyn, Dr. M. Gunther, and Mr. R. A. Green. I am indebted to the Medical Research Council and the Darwin Trust for financial assistance.

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APPENDIX

Data giving Birth Ranks and Maternal Ages at Births of 1031 *Individuals in* 217 *Sibships*

The serial numbers of the sibships and sexes of the affected individuals are given in the first two vertical columns.

The maternal ages at the births of the sibs are given at the top of the page.

Affected individuals (M) given in bold type.

Normal individuals (N) given in ordinary type.

Serial number	Sex	Maternal Age.																															
		17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
1	f											4		5		6		7		8		10		11	12		13		14				
2	m					1		2				3		4																			
3	m			1		2			3																								
4	m																					7											
5	m					1		2																									
6	m, f								2		4			6							8		9			10							
7	m					1	2		3		4		5			6			7		8		9				11						
8	m															1	2		3		4		5		6								
9	m																4		5											7			
10	m					1							2														3						
11	f															1	2						3										
12	f							1	2		3			5		6			8			9					12					13	
13	f																2		3														
14	m														2				3														
15	f										2											3											
16	f																				1		2		3			4					
17	m							1		2		3			4			5		6			7							9			
18	f			1			3					4			5	6		7		8				9			10						
19	m	1	2	3	4							5									10	11		12			13						
20	m, f									1	2	3				4		5		6			7										
21	f									1		2		3													5						
22	m								1					2		3				4		5						6					
23	m																			1													
24	m																1		2	3		4								6			
25	f																								1								
26	f							1		2				3					5				6										
27	f								1		2		3					5		6												8	
28	f												1		2				4						7			8					
29	m											1		2		3								5						6			
30	m									1						2								3		4			5				

31	f					2		3					5			6				7									8						
32	m																									1									
33	f				1			2			3			4		5												7		8				9	
34	m																									1				2					
35	m										1			3			5					6			7					9					
36	m																														2				
37	f																																		4
38	f										1								2																
39	m													1								2				4									
40	m																1						2												
41	f															1			2										3						
42	m						3										5									7				8					10
43	f							1		2	3			4			6	7		8				9											
44	m						1	2		3		4		5						6															
45	m							1			2			3			4		5			6			7										
46	m			1							4	5		6					7									8							
47	m																			1					2					4					
48	m	1				2		3			5		6			7		8	9			10						12		13		14			15
49	m												1					2					3			4						5			
50	m													1				2							4										
51	m									1		2		3		4						5	6												
52	m																					1			2						4				5
53	m																		1				2						3						
54	f							1					2																						
55	f				1			2			3																								
56	f																		3	4					5			7							
57	f					2		3			4	5		6			7		8			9							11					12	
58	f									1	2							3												4					
59	m						1			2			3				5	6				7		8											
60	m			1			2			3	4							5	6			7			8	9			10						
61	m																		1			2				3									
62	f	1				2					3																								
63	f			1	2		3			6		7	8			9				11		12			14					15	16				
64	m																	1		2		4		5				6							
65	m					1	2							4																					

Maternal Age—(*continued*)

Serial number	Sex	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
66	m																				1					2							
67	f												1		2					3				5		7				8			
68	f														1		2									4							
69	f									1		2			3								4					5					
70	f										1	2				3		4															
71	f				1		2						4				5		7			8				10							
72	f		1						3									4		5			6		7								
73	m					1		2			3													4									
74	f											1											2		3								
75	m														3		4		5		6		7			8							
76	m												1		2			3				4						6					
77	m							1	2			3			4		5		6			7		8		9							
78	m																	6		7		8	9			10							
79	f												2			3		4	5					6					7				
80	f								1				2		3	4		5					6										
81	f																										2						
82	m																							12		13							
83	m							1		2		4		5				6															
84	m																		3		5		6		7								
85	m			1			2		3		4	5																					
86	m										1					2				3		4		5									
87	f											2																					
88	f			1		2	3		4			5			6		7			9			10		11								
89	f																	1	2						3								
90	f													2		3				4													
91	f							2	3		5				7	8				10	11		12		14			15			17		
92	m														1			2															
93	f													1																			
94	f					1										2					4			5					6				
95	m																												8				

96	m								1		2			3		4		5			6		7							8			9	
97	m							1		2		4				5				6	7	8										9		
98	m					2																				4								
99	m							1		2		3			4	5	6							7										
100	m					1					5				6			7				8				9		10						
101	m															1		2				3	4							5				
102	m					1					3			4			5																	
103	m	1			3						5			7		8				6 6						9								
104	m															1	2									3								
105	m					1			2											3		4		5									6	
106	m															2												3						
107	m			1		2			3		5			6		7		9			10		11					13						
108	f					1			2	3		4				6				7	8			9				11				13		
109	m							1	2												4							5						
110	m																										12		13			14		
111	m				1			2																		7								
112	f				3	4					6					7				8			10			11								
113	m									1		2															3							
114	f																						1											
115	m													1			2						3											
116	m										1					3						4												
117	f									1					2			3																
118	m		2		3										5					7							11							
119	f		1			2																												
120	m	1	2						3			4			5		6			7		8												
121	m																	1				2		3										
122	m																												9					10
123	m											1				2		3		4						6								
124	f													2			3				4													
125	m				2					4				5			7	8				10					12						13	
126	m		1		2	3			4	5						6	7			8	9			10										
127	f																1	2		3				4										
128	m								2					3			4			5		6					7						8	
129	m			1					3					5				8				9		10										
130	m														2		3																	

Maternal Age—(*continued*)

Serial number	Sex	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
131	m															1			2							3							
132	f													2		3			5									8					
133	m														2			3			5			6		7		8				9	
134	m									2				4		5										10							
135	f																						7	8		9		10		11			
136	m															5		6		7		8	9			10							
137	f							1					2		3			4		5	6		7		8								
138	f					I				2		3			4						5			6			7						
139	m, m														1		2		3			4			5		6		7				
140	f							1				2			3									4					5				
141	m											1	2																				
142	f				1					2																							
143	m									1												2					3						
144	m, m										3							6		7	8				{9 {9					10			
145	f						1	2	3	4		5		6		7		8															
146	m										1	2																					
147	m				2				3	4		5										6											
148	m							2		3		4			6		7			8	9												
149	m																					5			6		7						
150	m																2			3					4				6				
151	f		1		2		3		4		5				7	8		9			10		11						16			17	
152	m																	[illegible]								4		5					
153	f						1				2			3					4														
154	f										1			3				4			6			7					8				
155	m									1		2							4						5								
156	f						4			6			7	8		9		10					13							17			
157	m																				11		12	13		14							
158	m									3			6		7					8		10				12		15					
159	f														3			4					5										
160	f									1	2		3		4				5	6				7									

161	m												1												2				
162	f											1							2										
163	m															1						2		3					
164	f																	5		6		7							
165	m														1			3	5		6				8				
166	m						1		2						5						7				8				
167	m						1	2									3												
168	f																						2		4				
169	m																	4		5			6						
170	f								1			2																	
171	f												1		2			3											
172	m										1		2		3														
173	m						1		2		3				4			5									6		
174	m		1	2										6			7							8		9			
175	m									2		3						4											
176	m		1								4		5		6		7		8										
177	f, f					1	2							4			5												
178	m		1		2		3		4					6				9											
179	m							1			2		3			4		5											
180	m						1	2		3		4		5		6			8	9					12		13		14
181	m									2			3					5					7			8			
182	m	1		3		4		5			6				7		8												
183	f								1		2		3		4		5		6		7			8			{9 9		
184	m																								3				
185	m		2				4			5		6						8			10			11			13		14
186	m															3					4								
187	m					1		2		3		4			6		8		9			10		12					
188	m																	1		3		4				6			
189	m										1		2		3				4		5			7				9	
190	m									4					5			6											
191	m					1				3	4				5			7											
192	f																				1								
193	f										1	2							3	4									
194	m			2						5		7			9		10		11			12	13						
195	m							1							2														

Maternal Age—*(continued)*

Serial number	Sex	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
196	m						1			3			7				9																
197	m					1		2	3		4		5		6							7											
198	m, f															3			4				5						6				
199	f								1	2							4	5							7								
200	f																					1			2			3					
201	m														1		2		3			4											
202	f												1						2								3						
203	m						1																										
204	m													1								3											
205	f												1			3	4				5				6							8	
206	m			1																													
207	f																													1			
208	f															1				4		6	7				8						
209	f																		1						2								
210	m									1		2									3			4									
211	m														9	10						13						14				15	
212	f												1		2	3																	
213	m											4			5		6			7													
214	m																		2		3						6						
215	m				1		2			3										8		9		10		11		12			13		14
216	f															5	6	7			10						14	15					
217	m, m																		2												3 3		
		1	3	6	14	17	16	26	42	39	41	40	38	41	51	42	48	45	42	49	34	28	32	27	21	18	12	11	8	8	6	1	—
		—	—	3	1	3	4	4	1	—	3	6	3	2	2	4	6	6	9	13	8	15	14	14	18	16	23	12	12	7	8	6	1

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The Kinetics of Hæmoglobin IV—*General Methods and Theoretical Basis for the Reactions with Carbon Monoxide*

By F. J. W. ROUGHTON

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GENERAL INTRODUCTION

Some years ago, Hartridge and Roughton published four papers (1923–1927) upon the kinetics of the rapid reactions between oxygen and hæmoglobin. Following on this work an investigation has been made of the corresponding reactions between carbon monoxide and hæmoglobin, and also of the reactions in which carbon monoxide and oxygen both compete for union with hæmoglobin. According to the classical paper of Douglas, Haldane and Haldane (1912) the hæmoglobin-carbon monoxide reactions should only differ from the hæmoglobin-oxygen reactions in the numerical value of the mass action constants. It was therefore expected that the kinetic study of these sister reactions would contribute towards a fuller understanding of the reactions between oxygen and hæmoglobin. Furthermore, a knowledge of the rates of the carbon monoxide reactions is of service, not only in working out the factors involved in the passage of O_2 into the red blood corpuscle (Roughton, 1932), but also in interpreting the measurements of the carbon monoxide diffusion constant of the lung by the Bohr-Krogh method. Such study is likewise of value for the full understanding of other uses of carbon monoxide as a "physiological reagent," *e.g.*, determination of blood volume and distribution, indirect measurement of O_2 pressure in arterial blood, coal gas poisoning, etc. Lastly, the similarity between the kinetics of the reactions of O_2 and CO with hæmoglobin and the kinetics of numerous enzyme processes, as set forth especially by J. B. S. Haldane in his recent book (1930), makes this problem well worth working out from the viewpoint of enzyme chemistry.

In several respects the carbon monoxide reactions of hæmoglobin are easier and more satisfactory to study than the oxygen reactions :—

(i) The rates are slower, and hence easier to handle experimentally.

(ii) Larger variations in concentrations of the reagents are feasible—hence any kinetic equation can be tested in a more rigorous manner.

(iii) Smaller corrections are needed for the back reactions.

(iv) Smaller quantities of material are needed.

(v) The results can be checked by several independent methods.

In the work described below the velocity of three separate reactions of hæmoglobin has been measured—

(*a*) The combination of carbon monoxide with reduced hæmoglobin (Part V).

(*b*) The displacement of oxygen from combination with hæmoglobin by carbon monoxide.

(*c*) The displacement of carbon monoxide from combination with hæmoglobin by oxygen.

Reactions (*b*) and (*c*) are treated in Part VI.

In the present paper a summary is given of the general experimental procedure and of the physico-chemical basis of the several reactions, though special points will, of course, be dealt with in the more specialized papers. Finally, Part VII gives a preliminary account of the reactivity of freshly formed hæmoglobin compounds—an inquiry suggested by certain anomalous findings in Part VI.

Unless otherwise stated, sheep hæmoglobin was used throughout. The recent work of Geiger (1931) and Brinkman, Wildschut, and Wittermans (1934) suggests that in some species of mammalian bloods there is more than one kind of hæmoglobin present. According to Brinkman (private communication) this is probably not so in sheep's blood, at any rate, if the course of hæmoglobin denaturation in alkaline solution is used as criterion.

The solutions were simply prepared by laking the whole blood, and diluting with appropriate amounts of standard buffer solutions. No purification of the hæmoglobin was attempted, for this would have increased the labour greatly, and it was thought unlikely that the other substances present in blood, when diluted 100-fold or more, would affect the kinetics of the processes under study. This is confirmed by the recent finding of Hall (1934) who has shown that in M/15 phosphate buffer, p_H 6·8, dilute solutions of laked whole blood give the same dissociation curve as solutions of hæmoglobin purified by Adair's method.

Owing to the lability of sheep's hæmoglobin, all experiments were as far as possible completed within 24 hours. This requirement made it impossible to vary the conditions, *e.g.*, concentration of reagents, over as wide a scale as might from a physico-chemical point of view have been wished.

Notation

The following symbols are used throughout this series of papers :—

1 millimol = 1 mM = 22·4 cc.

[COHb] = mM CO combined with Hb per litre of solution.

$[O_2Hb]$ = mM O_2 combined with Hb per litre of solution.

[Hb] = concentration of reduced hæmoglobin per litre expressed as mM of additional O_2 (or CO) which would have to combine to saturate the hæmoglobin completely.

$$\%\mathrm{COHb} = 100 \times \frac{[\mathrm{COHb}]}{[\mathrm{COHb}] + [\mathrm{O_2Hb}] + [\mathrm{Hb}]}.$$

$$\%\mathrm{O_2Hb} = 100 \times \frac{[\mathrm{O_2Hb}]}{[\mathrm{COHb}] + [\mathrm{O_2Hb}] + [\mathrm{Hb}]}.$$

[CO] = mM dissolved CO per litre.

$[O_2]$ = mM dissolved O_2 per litre.

1—GENERAL EXPERIMENTAL METHODS

A—*The Rapid Flow Method*

The method of Hartridge and Roughton for measuring the velocity of the reactions of hæmoglobin is as follows.

The two solutions which are to react with one another are placed in separate bottles and driven thence via separate leads into a mixing chamber. The mixed fluid emerges from the mixing chamber into an observation tube, and the concentration of the various hæmoglobin compounds in the streaming fluid is measured at various positions along the tube by means of the Hartridge reversion spectroscope. Such readings together with a knowledge of the rate of streaming of the fluid along the observation tube, and of the total concentration of each reagent, give all the data necessary for measuring the velocity of the reaction. Diagrams of the arrangement may be found in previous papers of Hartridge and Roughton.

Table I shows the time range, and the nature and concentration range of the reagents used in studying the three reaction velocities.

The following details also require mention :—

(i) *Mode of Preparation of the Reagents*—Earthenware bottles of 5 to 20 litre capacity were used.

(*a*) *Solutions of Dissolved* CO *and/or* O_2—The bottle was half-filled with water or buffer solution, the residual air evacuated and replaced by the appro-

Table I

	(*a*) $CO + Hb \rightarrow COHb$	(*b*) $CO + O_2Hb \rightarrow$	(*c*) $O_2 + COHb \rightarrow$
Time scale	1·00 to 0·01 sec	30·0 to 0·1 sec	600 to 10 sec
Bottle A—			
Nature of reagent	Dissolved CO	Dissolved CO	Dissolved O_2
Concentration	0·100 to 0·001 mM	0·600 to 0·025 mM	1·5 to 0·3 mM
Bottle B—			
Nature of reagent	Reduced Hb	O_2Hb + dissolved O_2	COHb
Concentration	0·100 to 0·003 mM	O_2Hb 0·100 to 0·002 mM Dissolved O_2 0·600 to 0·050 mM	10 to 0·005 mM

priate gas mixture. Five minutes rolling sufficed to bring the liquid and gas phase into equilibrium. The amount of dissolved gas was either calculated from the composition of the gas phase and the appropriate solubility coefficients, or else was measured by modifications of the usual methods of blood gas analysis.

(*b*) *Oxyhæmoglobin Solution*—A suitable amount of laked, oxygenated blood was added to buffer solutions equilibrated with various partial pressures of oxygen.

(*c*) *Carboxyhæmoglobin Solution*—Whole blood, laked with 4 parts of water, was equilibrated with a low partial pressure of CO, so that the hæmoglobin should be fully saturated with CO, without there being an appreciable amount of the latter in simple physical solution. One part of this COHb solution was then quickly mixed with 200–300 parts buffer solution, of very low [O_2].

(*d*) *Reduced Hæmoglobin Solution*—Whole or laked blood was reduced by repeated evacuation and shaking with nitrogen at about 30° C. It was then mixed with a suitable large volume of O_2-free buffer solution anaerobically.

For experiments at $p_H > 10$, the ammonium sulphide method of our previous paper (Hartridge and Roughton, 1925) was sometimes used.

(ii) *Observation Tube*—Tubes of diameter 6 to 13 mm served for the higher Hb concentrations, whilst for lower ones, the type shown by Hartridge and Roughton in fig. 2 of their 1926 paper was used.

(iii) *Intermediate Volumes*—For the slower reactions (half period > 1 sec) vessels of capacity 10 to 3000 cc were interpolated between the mixing chamber and the observation tube (Hartridge and Roughton, 1926, fig. 1).

(iv) *The Reversion Spectroscope*—For the displacement of O_2 from Hb by CO, and *vice versa*, the ratio of [COHb] to [O_2Hb] in the streaming fluid was measured in the ordinary way.

In the combination of CO with reduced Hb, it was, however, necessary to e the [COHb] : [Hb] in the streaming fluid. This was done in a manner similar to that adopted by Hartridge and Roughton (1923) for measuring the ratio $[O_2Hb]:[Hb]$, the only essential difference being that the auxiliary trough which in the previous investigation contained COHb, in the present one contained O_2Hb solution. Otherwise the procedure for calibration, estimation, etc., was so like that already described for the $[O_2Hb]:[Hb]$ measurement that reference to the earlier paper will supply any further details needed.

In the present work, readings can be got from 15% COHb to 100% COHb, whereas in the $[O_2Hb]:[Hb]$ work, the absorption bands, owing to blurring, could not be measured up at lower values than 30% O_2Hb. The best accuracy is found between 30 and 70% COHb, but even in this range the accuracy is only half as good as in the [COHb] : $[O_2Hb]$ estimation in solutions containing no reduced Hb. At the end of Part V a more accurate, though more elaborate, spectroscopic method is described for measuring [COHb] : [Hb] ratios.

B—*The Shaking Method*

Under suitable conditions, both the displacement of O_2 from combination with Hb by CO, and also the reverse reaction, have a half-period of more than 30 sec. Reactions so slow as this can also be followed by a simpler method, *i.e.*, by shaking the Hb solution violently with appropriate gas phases, and then after varying periods stopping the shaker and examining the percentage of COHb in the liquid with the reversion spectroscope. Comparisons with the rapid flow method showed satisfactory concordance.

Besides that of simplicity, the shaking method has the further advantage of requiring only very small amounts of blood, *i.e.*, of the order of 0·1 cc or less. Applications of it are given in Part VI, p. 473. Unfortunately it has not so far proved applicable to the CO + Hb reaction rate.

Two other methods have also been applied to the special CO + O_2Hb reaction. These, too, are described in Part VI of this series of papers.

2—GENERAL PHYSICAL CHEMISTRY OF THE HÆMOGLOBIN REACTIONS

A—*The Reaction of Carbon Monoxide (or Oxygen) with Reduced Hæmoglobin*

The simplest view of the reaction from the mass-action standpoint is that originally put forward by Hüfner, *i.e.*, that

$$1 \text{ mol CO (or } O_2) + 1 \text{ mol Hb} \rightleftharpoons 1 \text{ mol COHb (or } O_2\text{Hb)}.$$

This leads to the velocity equation

$$d\,[\mathrm{COHb}]\,/dt = l'\,[\mathrm{CO}]\,[\mathrm{Hb}] - l\,[\mathrm{COHb}], \tag{1}$$

and to the equilibrium equation

$$\frac{[\mathrm{COHb}]}{[\mathrm{CO}]\,[\mathrm{Hb}]} = \frac{l'}{l} = \mathrm{L}, \tag{2}$$

the latter leading to a dissociation curve of the rectangular hyperbola type. These equations would only be *prima facie* valid if the molecular weight of hæmoglobin did only combine with 1 gm mol of CO (or O_2), *i.e.*, if the molecular weight of hæmoglobin was, as Hüfner supposed, 17000.

Actually, however, the work of Adair, Svedberg, and others has shown that the molecular weight of the respiratory pigments $= 17000n$, where

$n = 2$ for muscle hæmoglobin (Theorell, 1934),

$n = 4$ for mammalian blood hæmoglobin (Adair, 1925, Svedberg),

$n > 50$ for the analogous case of the hæmocyanins (Svedberg, 1933).

This, as Adair (1925) pointed out, would suggest the existence of three types of pigment compound. (i) In which none of the gas-combining groups (*i.e.*, " gas-groups " for short) are combined with CO or O_2. (ii) In which all of the gas-groups are combined with CO or O_2. (iii) In which *some* but not all of the gas-groups are combined.

Hartridge and Roughton (1925) showed that simple equations of the Hüfner type (*i.e.*, (1) and (2) above) should hold, whatever the value of n, provided that the chance of a gas molecule combining with the hæmoglobin molecule was just proportional to the concentration of dissolved gas and the number of free gas-groups in the molecule, and that the chance of dissociation of a gas molecule from combination with hæmoglobin depended only on the number of gas-groups occupied. A similar deduction was made independently by Adair (1925). Such a proviso is essentially similar to that shown by Langmuir to hold good in reactions with heated metallic surfaces. The type of reaction in question may hence be referred to as a " Langmuir " type.

Experimentally the simple equations have been found to hold for :—

(i) Equilibria for muscle hæmoglobin (Theorell, 1934) ; mammalian hæmoglobin spasmodically, especially if the blood has received rather strenuous handling (Barcroft and Roberts, 1909 ; Hartridge and Roughton, 1925 ; Forbes, 1931) ; dialysed limulus hæmocyanin (Redfield, 1930).

$Hb_4(CO) + CO \rightleftharpoons Hb_4(CO)_2$.

$$\text{Velocity} = l'_2[CO][Hb_4(CO)] - l_2[Hb_4(CO)_2].$$
$$\text{Equilibrium constant } L_2 = l'_2/l_2. \tag{5.2}$$

$Hb_4(CO_2) + CO \rightleftharpoons Hb_4(CO)_3$.

$$\text{Velocity} = l'_3[CO][Hb_4(CO)_2] - l_3[Hb_4(CO)_3].$$
$$\text{Equilibrium constant } L_3 = l'_3/l_3. \tag{5.3}$$

$Hb_4(CO)_3 + CO \rightleftharpoons Hb_4(CO)_4$.

$$\text{Velocity} = l'_4[CO][Hb_4(CO)_3] - l_4[Hb_4(CO)_4].$$
$$\text{Equilibrium constant } L_4 = l'_4/l_4. \tag{5.4}$$

Let $[CO] = \alpha$, initial $[Hb] = \beta$.

The final equilibrium equation then runs

$$\frac{\%\text{COHb}}{100} = \frac{L_1\alpha + 2L_1L_2\alpha^2 + 3L_1L_2L_3\alpha^3 + 4L_1L_2L_3L_4\alpha^4}{4(1 + L_1\alpha + L_1L_2\alpha^2 + L_1L_2L_3\alpha^3 + L_1L_2L_3L_4\alpha^4)}. \tag{6.1}$$

At very high values of α, (6.1) simplifies down to the equation

$$\frac{\%\text{Hb}}{100} = \frac{1}{4L_4\alpha}. \tag{6.2}$$

Exactly similar equations have been previously given for the O_2-Hb equilibrium. By choosing the values of L_1, etc., suitably, the *whole* dissociation curve can be very closely fitted, but, as has been fully realized in previous papers, such a test has no rigour for many different sets of values of L_1, etc., all give an equally good fit.

At first sight the situation as regards the velocities looks even more unpromising. For there are not only four unknown forward velocity constants l'_1, l'_2, l'_3, l'_4, but also four unknown backward velocity constants to be taken into account. The latter, however, are relatively so small that the terms containing them can be neglected except very near the end of the reaction; furthermore, it is possible under restricted conditions to devise tests, the stringency of which does not depend upon an independent knowledge of l'_1, l'_2, l'_3, and l'_4.

(*a*) If $[CO] \gg \beta$, so that [CO] remains practically constant throughout the extent of the reaction under consideration, then if the back reaction terms are neglected it follows that

$$[\text{COHb}] \text{ at time } t = \beta[1 - (A_1e^{-l'_1\alpha t} + A_2e^{-l'_2\alpha t} + A_3e^{-l'_3\alpha t} + A_4e^{-l'_4\alpha t})], \tag{6.3}$$

where A_1, A_2, etc., are constants with values depending only on the value of l'_1, l'_2, etc.

A similar equation has been given by Millikan (1932) for the rate of dissociation of oxyhæmoglobin on the intermediate compound hypothesis.

From equation (6.3) it follows that—

(i) If [CO] is varied but kept in great excess of β, the time taken to reach any given COHb per cent should be inversely proportional to [CO].

This is verified experimentally in Part V.

(ii) Over the very early stages of the reaction $d\,[\mathrm{COHb}]\,/dt$ should equal $l'\,[\mathrm{CO}]\,[\mathrm{Hb}]$ roughly, *i.e.*, the Hüfner equation should hold good even though the values l'_1, l'_2, l'_3, l'_4 are not known. Experiments in Part V show that this equation is roughly obeyed, even beyond the early stages of the reaction in many cases.

(*b*) Let $[\mathrm{Hb}_4] = y_0$, $[\mathrm{Hb}_4\,(\mathrm{CO})] = y_1$, $[\mathrm{Hb}_4\,(\mathrm{CO})_2] = y_2$, etc., and $y = y_1 + 2y_2 + 3y_3 + 4y_4$.

Then [CO] at time t = ([CO] at time 0) $- y = \alpha - y$.

Therefore rate of reaction $\mathrm{CO} + \mathrm{Hb}_4 \rightarrow \mathrm{Hb}_4\,(\mathrm{CO})$ is given by

$$\frac{dy_0}{dt} = -\,l'_1 y_0\,(\alpha - y), \tag{7.1}$$

the back reaction term being neglected, whence

$$\frac{d\,(qy_0)}{d\,(t/q)} = -\,l'_1 q y_0\,(q\alpha - qy), \tag{7.2}$$

where q is any multiplier.

From equations (7.1) and (7.2) it follows *rigorously* that the effect of increasing both [CO] and [Hb] q-fold, should be to increase $d\,[\%\mathrm{Hb}_4\,(\mathrm{CO})]\,/dt$ q-fold over the whole course of the reaction, whatever the shape of the reaction velocity curve. By similar arguments the same should apply to each of the other intermediaries $\mathrm{Hb}_4\,(\mathrm{CO})_2$, $\mathrm{Hb}_4\,(\mathrm{CO})_3$, $\mathrm{Hb}_4\,(\mathrm{CO})_4$. From this it clearly follows that %COHb plotted against time measured in units of 1 second, should give a curve identical with that of %COHb plotted against time measured in units of $1/q$ second, if in the latter case [CO] and [Hb] are both increased q times.

This important deduction is verified in Part V. In this instance it will be found that an equation of the type $d\,[\mathrm{COHb}]\,/dt = k'\,[\mathrm{CO}]^n\,[\mathrm{Hb}]$, where $n \geq 2$ fails entirely. Failure in less striking degree also occurs in the other two tests already mentioned in this section.

Although these kinetic experiments do serve to discriminate against the Hill equation, they do not give any decision as between the general intermediate compound hypothesis, and the simple Langmuir-Hüfner mechanism, for the latter, as can readily be seen, yields deductions which are identical with those given above. From the kinetic standpoint it has, indeed, so far proved difficult to distinguish between the last two mechanisms; but since dissociation curve data, in dilute solutions of mammalian blood, pronounce definitely against the Hüfner-Langmuir mechanism, it would seem best, at present, to accept the general theory of intermediate compounds as the best working hypothesis.

B—*The Displacement of* O_2 *from Combination with Hæmoglobin by* CO *and* vice versa

The equilibrium of this reaction has been studied extensively both by gasometric and by optical methods. If the sum of $[CO] + [O_2]$ is high enough for [Hb] to be practically negligible, all observers are agreed that the equilibrium can be expressed by the simple equation

$$\frac{[O_2]\,[COHb]}{[CO]\,[O_2Hb]} = M. \tag{8}$$

The constant M is practically independent of p_H, salt content, laking of the blood, and purification of the hæmoglobin. It has a temperature coefficient of about 1·3 per 10° C, and is very greatly reduced by strong light intensity.

If the hæmoglobin is practically saturated with CO and/or O_2, there should, on the intermediate compound hypothesis, be no appreciable amounts of compounds with less than *three* gas groups saturated. The natural view would then be that the process consists of a series of pairs of reactions as follows:—

$$\left.\begin{array}{l} Hb_4O_8 \rightleftharpoons Hb_4O_6 + O_2 \\ Hb_4O_6 + CO \rightleftharpoons Hb_4O_6\,(CO) \end{array}\right\} \tag{9.1}$$

$$\left.\begin{array}{l} Hb_4O_6\,(CO) \rightleftharpoons Hb_4O_4\,(CO) + O_2 \\ Hb_4O_4\,(CO) + CO \rightleftharpoons Hb_4O_4\,(CO)_2 \end{array}\right\} \tag{9.2}$$

$$\left.\begin{array}{l} Hb_4O_4\,(CO)_2 \rightleftharpoons Hb_4O_2\,(CO)_2 + O_2 \\ Hb_4O_2\,(CO)_2 + CO \rightleftharpoons Hb_4O_2\,(CO)_3 \end{array}\right\} \tag{9.3}$$

$$\left.\begin{array}{l} Hb_4O_2\,(CO)_3 \rightleftharpoons Hb_4(CO)_3 + O_2 \\ Hb_4\,(CO)_3 + CO \rightleftharpoons Hb_4\,(CO)_4 \end{array}\right\} \tag{9.4}$$

Consider the first pair of reactions given by (9.1). Experimental evidence suggests that the forward and backward reactions of the reversible process $Hb_4O_8 \rightleftharpoons Hb_4O_6 + O_2$ are both so much faster than either reaction of the second process $Hb_4O_6 + CO \rightleftharpoons Hb_4O_6(CO)$, that the former process must be practically at equilibrium throughout. As regards (9.1) we should therefore have :—

$$-\frac{d[CO]}{dt} = \frac{l'_4[CO][Hb_4O_8]}{K_4[O_2]} - m_4[Hb_4O_6(CO)],$$

where

l'_4, m_4 are the velocity constants of $Hb_4O_6 + CO \rightleftharpoons Hb_4O_6(CO)$,

and

K_4 is the equilibrium constant of $Hb_4O_8 \rightleftharpoons Hb_4O_6 + O_2$.

A similar series of equations applies to (9.2), (9.3), and (9.4), and these when summed together give the " overall " rate of combination of CO.

Let us see what would happen if the simplest Langmuir principles applied to these reactions.

In the reversible processes

$$Hb_4O_6 + O_2 \rightleftharpoons Hb_4O_8,$$

$$Hb_4O_4(CO) + O_2 \rightleftharpoons Hb_4O_6(CO),$$

$$Hb_4O_2(CO)_2 + O_2 \rightleftharpoons Hb_4O_4(CO)_2,$$

$$Hb_4(CO)_3 + O_2 \rightleftharpoons Hb_4O_2(CO)_3,$$

the velocity constants of the reactions from left to right would all be the same, *i.e.*, the velocity of each of these reactions would just depend on there being *one* free group available in the hæmoglobin molecule for O_2 to combine with, and would not depend on the nature of the molecules (whether O_2 or CO) which already occupy the other three groups.

The velocity constants of the reactions from right to left in the above reaction would not, on the contrary, be equal to one another, but would be proportional to the number of O_2 molecules combined in the hæmoglobin molecule. Thus, *e.g.*, the velocity constant for the reaction $Hb_4O_8 \rightarrow Hb_4O_6 + O_2$ would be taken as four times greater than the velocity constant for the reaction $Hb_4O_2(CO)_3 \rightarrow Hb_4(CO)_3 + O_2$, it being assumed that the chance of a hæmoglobin molecule which possesses four combined O_2 molecules dissociating off an O_2 molecule is four times greater than the chance of a hæmoglobin molecule which possesses only one combined O_2 molecule, the other three groups being occupied by CO.

Similarly for the reversible reactions

$$Hb_4O_6 + CO \rightleftharpoons Hb_4O_6\,(CO),$$
$$Hb_4O_4\,(CO) + CO \rightleftharpoons Hb_4O_4\,(CO)_2,$$
$$Hb_4O_2\,(CO)_2 + CO \rightleftharpoons Hb_4O_2\,(CO)_3,$$
$$Hb_4\,(CO)_3 + CO \rightleftharpoons Hb_4\,(CO)_4,$$

the velocity constants of the reactions from left to right would be taken as identical, but the velocity constants from right to left would stand to one another in the ratio of the numbers of combined CO molecules in the respective hæmoglobin compounds.

On such assumptions the "overall" rate for the whole process would be given by

$$\frac{d}{dt}[COHb] = \frac{l'_4\,[CO]\,[O_2Hb]}{4K_4\,[O_2]} - m\,[COHb], \qquad (10.1)$$

$$= \frac{m'\,[CO]\,[O_2Hb]}{[O_2]} - m\,[COHb],$$

where

$$m' = \frac{l'_4}{4K_4}, \qquad (10.2)$$

whence at equilibrium

$$\frac{[O_2]\,[COHb]}{[CO]\,[O_2Hb]} = \frac{m'}{m} = M. \qquad (11)$$

Equations (10.2) and (11) are tested in Part VI of the present series of papers, and found to be, within experimental error, valid.

No other theory has yet been found to give so simple and plausible an explanation of the experimental results in the present reaction. As a working hypothesis it may be tentatively concluded that, when not less than three out of the four "gas-groups" of the hæmoglobin molecule are combined with O_2 and/or CO, the reactions even in mammalian blood conform to the simple Langmuir principle, and that on this account the competition of CO and O_2 for combination with hæmoglobin can be expressed by very simple mass action equations.

The costs of the researches described in this paper and in the succeeding papers have in part been financed by the Medical Research Council, to whom I wish to express my thanks. I am also greatly indebted to Professor Hartridge for the loan of much of his private apparatus, and for his continual interest and indeed frequent assistance in many of the experiments.

SUMMARY

1—The rapid reaction velocity method of Hartridge and Roughton has been applied to the following reactions—

(*a*) the combination of CO with reduced hæmoglobin (Hb);

(*b*) the displacement of O_2 from combination with Hb by CO;

(*c*) the displacement of CO from combination with Hb by O_2.

General descriptions of the experimental technique are given, and the importance, theoretical and practical, of such measurements is emphasized.

2—The oxygen-hæmoglobin reaction is briefly reviewed from the physicochemical standpoint, with a view to the theoretical consideration of the carbon monoxide reactions.

3—Under certain conditions it is shown that the equation for the velocity of reaction (*a*) is identical on the Hüfner theory and on the intermediate compound hypothesis of Adair, but is quite different on the basis of Hill's equation.

These deductions are experimentally tested in Part V; the issue is favourable to the Adair hypothesis and not to the Hill theory.

4—Reactions (*b*) and (*c*) follow very simple equations both as regards their equilibrium point and their velocities. This is also difficult to reconcile with the Hill equation; it can, however, be brought into line with the Adair hypothesis, if the competition of CO and O_2 for combination with Hb obeys the same principles as those postulated (in simple cases) by Langmuir for the reactions between gases and heated metallic surfaces.

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The Kinetics of Hæmoglobin V—The Combination of Carbon Monoxide with Reduced Hæmoglobin

By F. J. W. ROUGHTON

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The theoretical aspects and importance of this reaction have already been discussed in the introductory paper of this series (Part IV) as have also the experimental methods in broad outline. It will be assumed here that the reader is familiar with that paper.

In the present paper will be found the experimental results of the tests suggested in Part IV, together with an account of the effect of p_H and temperature on the rate of combination of carbon monoxide with reduced hæmoglobin. It will be useful to describe first of all the results of a typical experiment at p_H 10, temperature 21° C, $[CO] = 0 \cdot 1$ mM, $[Hb] = 0 \cdot 06$ mM. These are plotted in fig. 1. It will be seen that the reaction is half completed in about 0·05 second and there is therefore no doubt that the apparatus is amply adequate for coping with the reaction, since in our previous papers we have shown that even reactions more than ten times as fast could still be accurately measured.

It was rather a surprise to find that the reaction between CO and Hb is about 10 times slower than the reaction between O_2 and Hb under similar conditions. The 200-fold greater "affinity" of CO for hæmoglobin must therefore be due to the extreme slowness with which COHb, as compared with O_2Hb, can dissociate. This conclusion is verified in Part VI, p. 484.

1—*Tests as to the Mechanism of the Reaction*

(i) *The Effect of Varying Excess* [CO], *at Constant Hæmoglobin Concentration.*—On either Hüfner's theory, or on the general intermediate compound hypothesis, the time taken to reach any given % COHb should be inversely proportional to [CO]. This should not, however, be so if Hill's equation applies to the kinetics of the reaction.

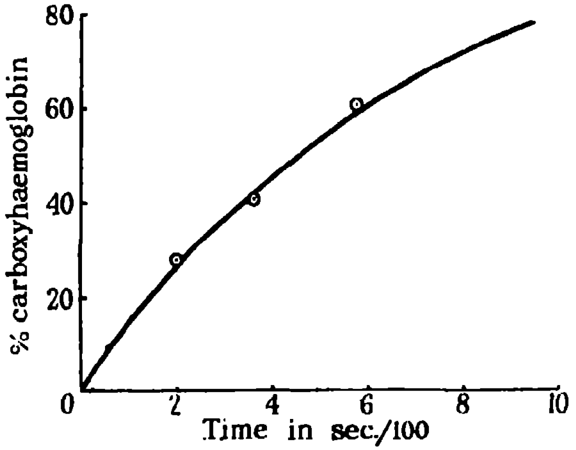

FIG. 1—Typical experiment on rate of combination of CO with hæmoglobin.

With the apparatus, as ordinarily used, it was not feasible to have [CO] in more than 10-fold excess over the hæmoglobin. In two experiments at 15° C now to be described, the following four solutions were prepared:—

(*a*) Reduced Hb solution in M/20 phosphate buffer p_H 6·6. [The blood was diluted to 1 in 40 approximately.]

(*b*) Reduced Hb solution of same strength in NH_3-NH_4Cl buffer p_H 10·0.

(*c*) Solution of dissolved CO in equilibrium with gas phase containing 1¼ atmospheres CO.

(*d*) Solution of dissolved CO in equilibrium with gas phase containing ⅓ atmosphere CO.

By mixing (*a*) first with (*c*), and then with (*d*), the effect of 3-fold variation in [CO] could be observed at p_H 6·6. Similarly at p_H 10·0 by using (*b*) in place of (*a*). The results of these four experiments are plotted in figs. 2*a*, 2*b*, in which the time scale is measured in units of 1/100 second for the stronger [CO], and in units of 1/33 second for the weaker [CO].

It is seen that the points in the (*a*) and (*c*) experiment when so plotted fall pretty closely on the same curve as the points in the (*a*) + (*d*) experiment, fig. 2*a*, over the first third of the process. Such also occurs with the (*b*) + (*c*) experiment and the (*b*) + (*d*) experiment, fig. 2*b*, thus verifying the deduction under test. The agreement in the latter experiment extends up to higher per cent COHb than would have been expected, for when 50% COHb is reached

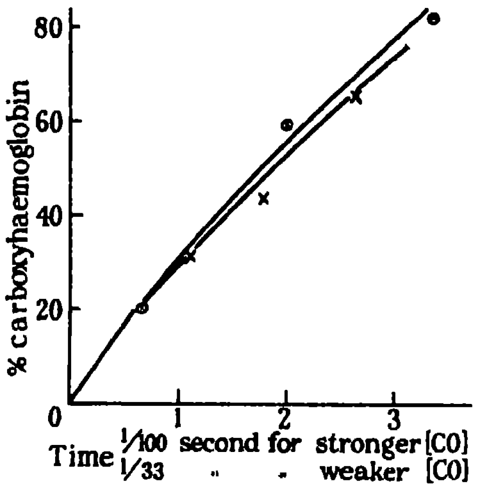

FIG. 2*a*—Effect of three-fold variation in [CO] at p_H 6·6, temperature 15° C. ⊙ strong [CO]; × weak [CO].

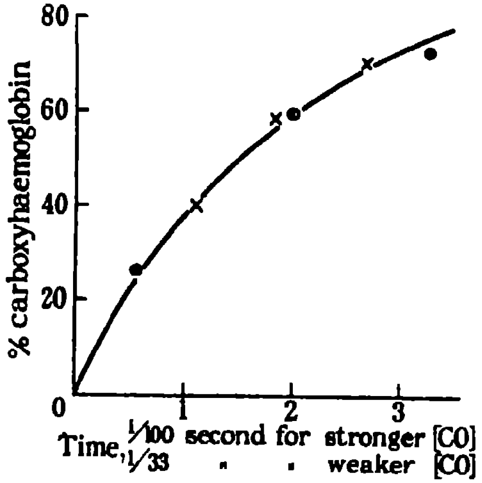

FIG. 2*b*—Effect of three-fold variation in [CO] at p_H 10·0, temperature 15° C. ⊙ strong [CO]; × weak [CO].

the [CO] in experiments with solution (*c*) will have dropped by 5% whereas the [CO] in experiments with solution (*d*) will have dropped 15%, so that in the latter case a distinct lagging of the %COHb might have been noticeable.

(ii) During the early stages of the reaction

$$d\,[\mathrm{COHb}]/dt \text{ should } = l'\,[\mathrm{CO}]\,[\mathrm{Hb}], \qquad (1.1)$$

both on the Hüfner theory and on the general intermediate compound hypothesis.

Equation (1.1), when integrated, gives

$$l't = \frac{1}{\alpha - \beta} \log \frac{\alpha - y}{\beta - y}, \qquad (1.2)$$

where

$$\alpha = [\mathrm{CO}] + [\mathrm{COHb}],$$
$$\beta = [\mathrm{COHb}] + [\mathrm{Hb}],$$
$$y = [\mathrm{COHb}],$$

whence $\log \frac{\alpha - y}{\beta - y}$ plotted against time should give a straight line relationship during the early stages of the reaction.

The results of fig. 1 are re-plotted in this manner in fig. 3. The points, indeed, fall on a straight line not only during the early stages but also far beyond. This was also observed in many other experiments, and so it seems legitimate to calculate an *empiric* velocity constant l' by equation (1.2). The word "empiric" is used to emphasize the fact that the general intermediate compound hypothesis is incapable of saying whether equation (1.2) ought or

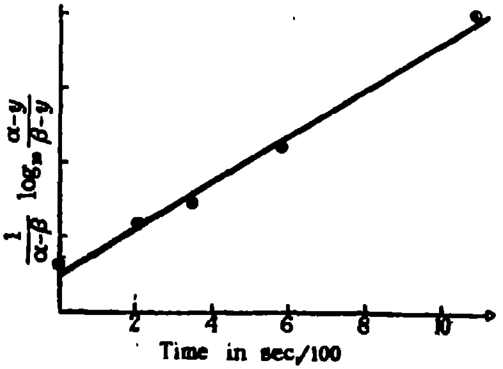

FIG. 3—Test of equation for velocity of reaction CO + Hb → COHb.

ought not theoretically to hold good beyond the early stages of the reaction. On the Hüfner theory, of course, equation (1.2) ought to be valid up to the point where the back reaction velocity becomes important.

(iii) By far the most important tests are those shown in fig. 4 at p_H 10, and at p_H 7·4. At each of these p_H's, the effect of varying the concentration both of CO and of hæmoglobin 32-fold was observed. According to Part IV this should alter the rate of change of the %COHb in the same ratio, viz., 32-fold, if the Hüfner theory or the general intermediate compound hypothesis hold good. At both p_H's the time scale is plotted in units of seconds/32 for the stronger concentrations, and in units of seconds for the weaker concentrations; the two sets of points for the two concentrations should both fit on the same curve. This they certainly do, within experimental error, at both p_H's.

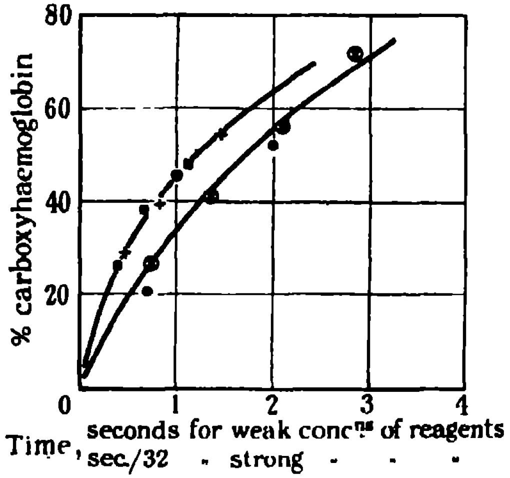

Fig. 4—Effect of thirty-two-fold variation in concentration of reagents upon rate of reaction CO + Hb → COHb at p_H 7·4 and 10. (i) p_H 10—■ weak concentration; + strong concentration. (ii) p_H 7·4—● weak concentration; ⊗ strong concentration.

The inadequacy of Hill's equation from the kinetic point of view is in these cases very striking. For on this view the reaction would proceed according to the equation. $Hb_n + nCO \rightarrow Hb(CO)_n$, where n lies between 2 and 3, and the effect of reducing Hb and CO each 32-fold should be to reduce the rate of increase of %COHb at least 1000-fold, instead of 32-fold as in the previous theory and *experiments*. The Hill theory, though still much used as an *empiric* basis for formulating oxygen-hæmoglobin equilibria, has thus, as already mentioned in Part VI, proved to be unserviceable in the kinetic field, and therefore unserviceable also as a guide to the mechanism of the reactions concerned.

The results of all these three tests are, on the contrary, in accord either with the simple Hüfner theory or with the general intermediate compound hypothesis. For reasons given in Part IV, it seems best at present to accept the latter as the best working hypothesis.

2—*Effect of* p_H, *Temperature, Light, etc.*

(i) p_H—The results of three experiments carried out on the same day (on Hb solution prepared from the same sample of blood) with identical concentrations of CO and Hb, but varing p_H, are plotted in fig. 5. It will be seen that the velocities at p_H 7·2 and p_H 5·6 are identical within experimental error, but that the velocity at p_H 10 is about 50% faster. This difference is about the same as the difference in rate of combination of O_2 + Hb at p_H 10 and p_H 5·6. In neither case is the difference large enough to make it possible to calculate with accuracy the value of the oxy-labile ionization constant of reduced hæmoglobin, as was found possible previously for oxyhæmoglobin (Hartridge and Roughton, 1923).

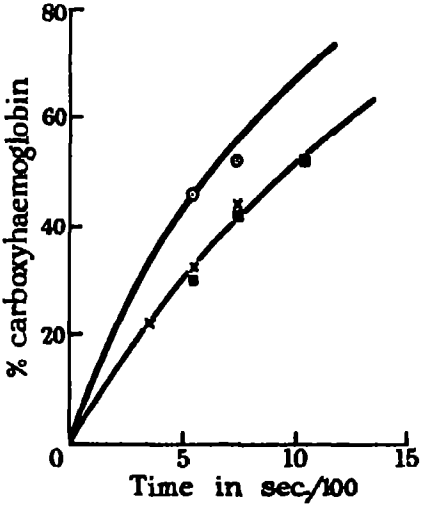

FIG. 5—Effect of p_H on velocity of reaction CO + Hb → COHb. ⊙ p_H 10; ■ p_H 7·2; × p_H 5·6.

Usually the buffer was all placed in the CO solution and the Hb solution previous to mixture was unbuffered (*i.e.*, about p_H 7·4) so that after mixture there must have been considerable changes in the ionisation of the hæmoglobin. That these changes are too rapid to have any effect upon the observed rate of the CO + Hb reaction was shown by controls in which the buffer was equally distributed between the CO and Hb solutions prior to their mixture in the reaction velocity apparatus.

(ii) *Temperature*—The values of l' at three different temperatures, $p_H = 7 \cdot 2$, as measured on the same day on hæmoglobin solutions prepared from the same sample of blood, are shown in Table I.

Table I

Temp. ° C	l'
7·2	118
17·7	252
33·3	747

Q_{10}, the temperature coefficient per 10° C, is therefore about 2. The values of Q_{10} in Table II were obtained at other p_H's, blood from *different* sheep being used in each case.

Table II

p_H	Q_{10}
6·6	1·6
5·6	1·8
5·6	1·7

(iii) *Light*—Haldane and Lorrain Smith (1896) discovered that the equilibrium of the reaction

$$\text{carbon monoxide} + \text{oxyhæmoglobin} \rightleftharpoons \text{oxygen} + \text{carboxyhæmoglobin}$$

is displaced markedly to the left by light. It is easy to show that the equilibrium of the reaction

$$\text{carbon monoxide} + \text{reduced hæmoglobin} \rightleftharpoons \text{carboxyhæmoglobin}$$

is also displaced to the left by light, simply by shaking a dilute solution of COHb in a tonometer full of N_2, which is placed in the path of a beam of strong light. The question then arises, is it the forward reaction, *i.e.*, the combination of carbon monoxide with reduced hæmoglobin, or the backward reaction, *i.e.*, the dissociation of carboxyhæmoglobin, or both reactions that are accelerated by light? To answer this question, as regards the reaction studied in this paper, the same arrangement was adopted as in the reaction between oxygen and hæmoglobin (Hartridge and Roughton, 1925). No certain difference could be detected in the rate of combination of carbon monoxide with hæmoglobin even when the light illuminating the reacting solution was varied 100-fold in intensity. The effect of light upon the equilibrium constant of the reaction CO + Hb $\rightleftharpoons$ COHb must therefore be due entirely to its accelerating influence upon the velocity of dissociation of COHb.

(iv) *Salt Concentration*—No experiments have yet been done on the effect of varying salt concentration (*i.e.*, of ions other than $\overset{+}{\mathrm{H}}$ or $\overset{-}{\mathrm{OH}}$) at constant p_{H}.

3—*A More Accurate Method of Measurement*

Recently we have had occasion to devise a more accurate, though more elaborate, method of measuring the per cent COHb at various instants of time after mixture : the principle is as follows :—

The solution of CO and the solution of reduced Hb meet together in mixing chamber M_1, fig. 6, mix and then travel through a variable length of tube and deliver into a second mixing chamber M_2 where the fluid meets and mixes with water saturated with O_2 (at one atmosphere pressure at least). Suppose that the length of time *t*, taken between the mixing chambers M_1 and M_2 is such that the %COHb reaches $x\%$, then within at most 1/500 second after mixing with the oxygenated water, the remaining $(100 - x)\%$ of reduced Hb becomes O_2Hb, so that in the early part of the observation tube leading out of M_2 the fluid is $x\%$ COHb and $(100 - x)\%$ O_2Hb. The value of x is determined by the reversion spectroscope in the ordinary way. The value of x remains constant for some distance as the spectroscope moves down O′ away from M_2, but after a certain point is reached the value of x begins to increase owing to the displacement of O_2 from O_2Hb by CO. The success of the method depends entirely upon the fact that the chemical combination of

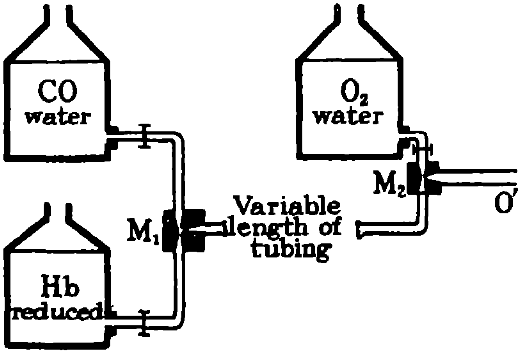

FIG. 6—More accurate method for measuring the rate of combination of CO with hæmoglobin.

the remaining reduced Hb with the dissolved O_2 is so very much more rapid than the combination with the remaining dissolved CO; to be certain of this it is best to work with as dilute a solution of hæmoglobin as possible so as to be able to have a correspondingly low concentration of CO. The value of t is determined by measuring the volume of tube in between M_1 and M_2, and the volume of fluid passing through M_1 per second. The value of x is measurable with at least twice as great accuracy as is possible in the former method, and further can be measured over the whole range from 0–100% COHb. With the 20-cm observation tube it is possible to work with a solution containing 0·5 cc blood per litre, which would be of obvious advantage in working with human blood or in studying the effect of species over a wide range of animals. The large effect of species upon the equilibrium constants of the hæmoglobin reaction does, indeed, make a similar investigation with the velocities well worth carrying out.

The method is also specially valuable for studying the rate of reaction of CO with hæmoglobin in the corpuscle, for in this reaction the suspension issuing from the first mixing chamber can be mixed with a solution, which not only contains excess of dissolved O_2 but also contains a laking agent, so that the final reading of COHb to O_2Hb is made in solution instead of in the corpuscle, in which, owing to the fuzzy character of the absorption bands caused by the scattering of light by the corpuscle walls, the readings are much more difficult and less accurate (Hartridge and Roughton, 1927).

Summary

1—Under similar conditions as to p_H, temperature, and concentration of reagents, carbon monoxide combines with hæmoglobin about 10 times more slowly than oxygen does. The greater affinity of CO for hæmoglobin is due to the fact that carboxyhæmoglobin dissociates far more slowly than oxyhæmoglobin does.

2—The results of three different quantitative tests suggest that the kinetics of the reaction conform to Adair's intermediate compound hypothesis, rather than to Hill's equation.

3—During the early stages of the reaction, the rate is given roughly by the equation

$$d\,[\mathrm{COHb}]/dt = l'\,[\mathrm{CO}]\,[\mathrm{Hb}].$$

4—l' is only slightly affected by p_H, and not appreciably by light.

5—The temperature coefficient of l' is about 1·8 per 10° C.

6—A new and more accurate method of measuring the rate of combination of CO with Hb is outlined.

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The Kinetics of Hæmoglobin VI—*The Competition of Carbon Monoxide and Oxygen for Hæmoglobin*

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INTRODUCTION

The experimental part of the research to be described below is given in two main sections. Of these Section 1 deals with the results obtained in measurements of the rate of displacement of oxygen from combination with hæmoglobin by carbon monoxide. Section 2 gives, in corresponding fashion, the results for the reverse reaction, namely, the displacement of carbon monoxide from combination with hæmoglobin by oxygen.

The theoretical aspects of these two reactions have already been dealt with in broad outline at the end of Part IV, with which it will be assumed that the reader is fully acquainted. Some further considerations, however, arise and these are given in Section 3 of the present paper.

The rate of displacement of oxygen from combination with hæmoglobin by carbon monoxide has now been measured in several different ways. The concordance so found is of experimental, rather than of theoretical, interest and hence the account of these tests has been relegated to an Appendix.

SECTION 1—THE RATE OF DISPLACEMENT OF OXYGEN FROM COMBINATION WITH HÆMOGLOBIN BY CARBON MONOXIDE

A—*Formulæ used in Calculating the Results*

The experimental results upon the rate of this reaction have all been found to be consistent with equation (10.1) of Part IV, namely,

$$d\,[\mathrm{COHb}]/dt = \frac{m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]}{[\mathrm{O_2}]} - m\,[\mathrm{COHb}].$$

Over the first half of the reaction, the back reaction term, *i.e.*, $-\,m\,[\mathrm{COHb}]$, is negligible, and hence the equation reduces to

$$d\,[\mathrm{COHb}]/dt = \frac{m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]}{[\mathrm{O_2}]}. \tag{1}$$

In Hartridge and Roughton's first paper (1923, *a*) another equation, viz., $d\,[\mathrm{COHb}]\,/dt = k_2\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]$, was used with apparent success. In this early work, however, the $[\mathrm{O_2}]$ was not systematically varied, and was, in fact, nearly the same in most of the experiments; the results would therefore have been equally well fitted by equation (1).

If $\alpha = [\mathrm{CO}] + [\mathrm{COHb}]$,
$\beta = [\mathrm{COHb}] + [\mathrm{O_2Hb}] + [\mathrm{Hb}]$,
$\gamma = [\mathrm{O_2}] + [\mathrm{O_2Hb}]$,
$y = [\mathrm{COHb}]$,

equation (1) becomes

$$\frac{dy}{dt} = \frac{m'\,(\alpha - y)\,(\beta - y)}{(\gamma + y - \beta)}, \tag{2}$$

[Hb] being neglected since it is throughout so small compared with β.

Equation (2) when integrated over the time interval $(t_1 - t_2)$ gives

$$m'\,(t_1 - t_2) = \frac{\beta - \alpha - \gamma}{\alpha - \beta} \log_e \frac{\alpha - y_1}{\alpha - y_2} + \frac{\gamma}{\alpha - \beta} \log_e \frac{\beta - y_1}{\beta - y_2}. \tag{3.0}$$

Special cases—

(i) If γ is large compared with $(\alpha - \beta)$, (3.0) simplifies to

$$m'\,(t_1 - t_2) = \frac{\gamma}{\alpha - \beta} \log_e \frac{(\beta - y_1)\,(\alpha - y_2)}{(\beta - y_2)\,(\alpha - y_1)}. \tag{3.1}$$

(ii) If α and γ are both large compared with β, (3.0) simplifies to

$$m'(t_1 - t_2) = \frac{\gamma}{\alpha}\log_e\frac{\beta - y_1}{\beta - y_2} = \frac{\gamma}{\alpha}\log_e\frac{[O_2Hb]_1}{[O_2Hb]_2}. \tag{3.2}$$

(iii) If $\alpha = \beta$,

$$\frac{dy}{dt} = \frac{m'(\alpha - y)^2}{(\gamma + y - \beta)},$$

$$m'(t_1 - t_2) = \log_e\left(\frac{\alpha - y_1}{\alpha - y_2}\right) - (\beta - \alpha - \gamma)\left(\frac{1}{\alpha - y_1} - \frac{1}{\alpha - y_2}\right) \tag{3.3}$$

These equations were used in appropriate cases in the tests now to be described.

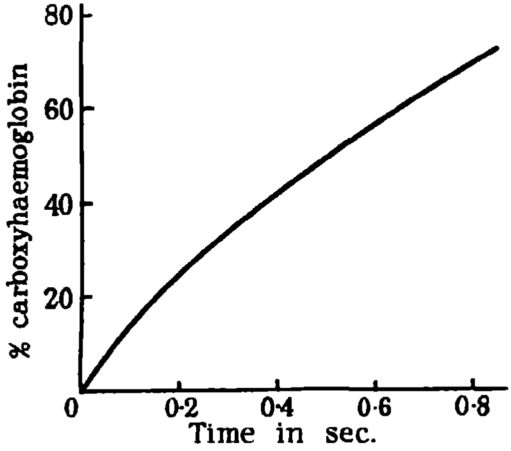

FIG. 1—Typical experiment on rate of reaction $CO + O_2Hb \rightarrow O_2 + COHb$, p_H 7·4, temperature 17° C.

B—*Results in a Typical Experiment*

The results of an experiment at p_H 7·8, temperature 21·5° C, are plotted in fig. 1. The solution in the two bottles, A and B, which delivered into the mixing chamber were as follows :—

Bottle A = water saturated with coal gas, practically free from dissolved O_2.

Bottle B = water saturated with air + 1 part of blood in 50 of water + p_H 7·8 buffer mixture.

In these experiments the conditions which lead to equation (3.1) are nearly enough satisfied and therefore $\log_{10}(\beta - y/\alpha - y)$ plotted against time should give a straight line if (3.1) is valid.

This was found to hold, within the limits of experimental error, both in the present experiment and in most of the other experiments on this reaction. It therefore follows that the various observations made in any one experiment give consistent values of m'. It is generally agreed, however, that a more searching test is to see whether m' remains constant when the concentrations of the various reagents are altered. The results of such tests will now be described in the next sub-section.

C—*Experimental Tests of Equation* $d[\mathrm{COHb}]/dt = \dfrac{m'[\mathrm{CO}][\mathrm{O_2Hb}]}{[\mathrm{O_2}]}$ *with varying Concentrations of the several Reagents*

(I) [CO], [O_2Hb] *varied;* [O_2] *kept approximately constant*—Tests on five different samples of sheep blood [(*a*), (*b*), (*c*), (*d*), and (*e*)] solution by the rapid flow method were done with varying concentration of CO and O_2Hb and at p_H's from 5·6 to 10·0. These are summarized in Table I.

Table I

Test	Temp ° C	p_H	α	β	γ	m	
(*a*)	15	7·4	0·070	0·092	0·345	3·4	3·65
	15	7·4	0·094	0·092	0·327	3·9	
(*b*)	17	7·4	0·079	0·067	0·223	3·1	3·43
	17	7·4	0·040	0·067	0·299	3·6	
	17	7·4	0·071	0·117	0·257	3·7	
(*c*)	21	6·3	0·060	0·067	0·223	4·6	4·75
	21	6·3	0·028	0·073	0·314	4·9	
	21	6·3	0·063	0·127	0·276	6·9 ?	
(*d*)	11	5·6	0·566	0·046	0·840	0·53	0·495
	11	5·6	0·0254	0·0021	0·930	0·46	
(*e*)	11	10·0	0·566	0·046	0·840	0·45	0·457
	11	10·0	0·0254	0·0021	0·930	0·50	

α, β, γ measured in millimols/litre.

In tests (*a*), (*b*), and (*c*) it was not practicable to vary [CO] and [O_2Hb] more than two-fold. Except in one instance the values of m' in any one test do not diverge by more than 10% from their mean value. This is within the experimental error of the method. The last two tests, viz., (*d*) and (*e*), show that the value of m' is not significantly altered by a much larger variation, viz., a 22-fold one, in the concentration of the reagents.

Equation (1) apparently, then, survives the test of varying [CO] and [O_2Hb]. Two further points may be noted :—

(i) Tests (*d*) and (*e*) provide good evidence against the kinetic applicability of Hill's equation, for in the first experiment of each test the rate at which %COHb increased should, on the Hill basis, have been $22^{2\cdot5}$, *i.e.*, 2000 times greater than the rate of increase in the second experiment. Actually the relative ratios were almost 22 to 1.

(ii) The values of m' in tests (*d*) and (*e*) are only 1/7 to 1/10 of those in (*a*), (*b*), and (*c*). This is in part due to the lower temperature in these tests, but must be caused chiefly by individual differences in the hæmoglobin in the various samples of blood.

(II) $[O_2]$ *varied;* $[CO]$, $[O_2Hb]$ *kept constant*—In these circumstances, if equation (1) is valid, the time taken to reach any given percentage of COHb should be inversely proportional to $[O_2]$, provided the latter is present in excess. That this is so is shown by the results of Table II, all of which were done by the rapid flow method.

Table II

Test	Temp ° C	p_H	$\dfrac{[O_2] \text{ in first test}}{[O_2] \text{ in second test}}$	$\dfrac{t_{50} \text{ in second test}}{t_{50} \text{ in first test}}$
(*a*)	8	10·0	2·0	2·1
(*b*)	7	10·0	2·0	2·0
(*c*)	15	7·2	5·0	6·0
(*d*)	7	7·2	2·0	2·0

Each test consisted of two experiments with different $[O_2]$, but the same $[CO]$, $[O_2Hb]$ values.

A further set of tests was also done by the shaking method (Part IV). 5 cc of dilute O_2Hb solution were shaken in a 120 cc tonometer with the gas mixtures shown in Table III for periods of time directly proportional to the O_2 pressure in the gas phase in each experiment.

Table III

% CO in tonometer	% O_2 in tonometer	Time of shaking	% COHb at end of shaking period
		mins	
0·2	21	6·00	49·5
0·2	15·7	4·50	48
0·2	10·5	3·00	51
0·2	5·2	1·50	50
0·2	2·6	0·75	52

On the basis of equation (1), the same percentage of COHb should be reached in each test. Table III shows that, within the limits of experimental error, this is so.

A more detailed description is given of the final test made by the rapid flow method.

Six reagents were prepared, of these :—

Bottle X = 12 litres water equilibrated with a gas mixture containing 23 mm CO, 161 mm O_2.

Bottle Y = 12 litres water equilibrated with a gas mixture containing 23 mm CO, 4 mm O_2.

Bottle 1 = 4 litres of solution p_H 10 + 4 cc blood + gas mixture containing 45·5 mm O_2.

Bottle 2 = 4 litres of solution p_H 10, + 4 cc blood + gas mixture containing 87·0 mm O_2.

Bottle 3 = 4 litres of solution p_H 10, + 4 cc blood + gas mixture containing 160 mm O_2.

Bottle 4 = 4 litres of solution p_H 10, + 4 cc blood + gas mixture containing 440 mm O_2.

An observation tube 20 cm long was used and glass tubes with volumes ranging from 40 cc to 483 cc were inserted between the mixing chamber and the observation tube, according to the speed of the reaction being measured. Thus in Table IV—

Experiment *a* = bottle X + bottle 1. Intermediate volumes used 40 cc, 56 cc.

Experiment *b* = bottle X + bottle 2. Intermediate volumes used 56 cc, 87 cc.

Experiment *c* = bottle X + bottle 3. Intermediate volumes used 87 cc, 164 cc.

Experiment *d* = bottle Y + bottle 3. Intermediate volumes used 164 cc, 308 cc.

Experiment *e* = bottle Y + bottle 4. Intermediate volumes used 308 cc, 483 cc.

Table IV—α, β, γ in millimols per litre, p_H 10, temperature 12° C

Experiment	α	β	γ	m'
(*a*)	0·0149	0·00378	0·067	0·38
(*b*)	0·0149	0·00378	0·102	0·47
(*c*)	0·0149	0·00378	0·188	0·44
(*d*)	0·0149	0·00378	0·332	0·40
(*e*)	0·0149	0·00378	0·638	0·42
Average				0·42

The values of m' are seen to be the same within experimental error, even though $[O_2]$ was varied about 11-fold. This strongly supports the validity of equation (1) as regards the effect of varying $[O_2]$.

The results of Table IV are, on the other hand, particularly difficult to reconcile with Hill's equation. For, according to the latter, the rate of the reaction would be expected to vary as $1/[O_2]^n$. Taking $n = 2\cdot5$, this would mean that in experiment (*a*) of Table IV, the rate of the reaction should have been about 130 times faster than in experiment (*e*). Actually the rate was only 10·2 times faster, which agrees well with equation (1), on the basis of which a factor of 11·2 times would be expected.

The general result of all these tests is, therefore, that over the p_H range 6·0–10·0 the rate of the reaction is satisfactorily fitted by the equation

$$d\,[\mathrm{COHb}]/dt = \frac{m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]}{[\mathrm{O_2}]}.$$

Table V

Experiment	p_H	$\dfrac{m' \text{ at } p_H \text{ in question}}{m' \text{ at } p_H\ 10}$
(*a*)	5·6	1·32
	6·3	1·41
	6·9	0·86
	7·8	1·04
	10·0	1·00
(*b*)	7·4	1·37
	9·0	1·00
(*c*)	5·6	1·02
	10·0	1·00
(*d*)	7·4	0·85
	10·0	1·00
(*e*)	6·2	1·36
	10·0	1·00
(*f*)	6·3	1·65
	10·0	1·00

D—*Effect of p_H and Temperature upon m'*

(i) p_H—Experiments (*d*) and (*e*) of Table I were both done on the same sample of blood and show no appreciable effect of p_H upon m'. In the majority of such tests, however, the value of m' in the p_H range 5–7 was found to be somewhat greater (viz., about 30–40%) than in the range p_H 7–10. In Table V the ratio of m' at each p_H to its value at p_H 10 (on the same blood) is given, so as to make the comparison as easy as possible.

The relatively slight effect of p_H was unexpected and will be discussed again in § 3.

In most of the experiments the O_2Hb was simply dissolved in tap water (p_H 7·4) and the p_H was controlled by the addition of buffers to the CO solution. Controls in which the buffers were equally distributed between the O_2Hb and the CO solution gave the same value for m'. This shows that the rapid ionic changes which the hæmoglobin suffers when mixed with the various buffers are without influence on the observed value of m'. For further discussion of this matter, reference should be made to an early paper, by Hartridge and Roughton (1923, *b*, p. 423).

(ii) *The Effect of Temperature*—Hartridge and Roughton (1923, *a*) calculated the temperature coefficient for their velocity constant k_2, which was based on the kinetic equation $d\,[\mathrm{COHb}]/dt = k_2\,[\mathrm{CO}]\,[O_2\mathrm{Hb}]$.

Unfortunately, however, they did not know at that time that the velocity of the reaction was also inversely proportional to the concentration of dissolved O_2, and since there is no certainty that the latter was the same in their experiments at different temperatures, the temperature coefficient determined by them is not necessarily equal to that of m', the velocity constant of the present paper. Preliminary experiments, at p_H 5·6, show that the temperature coefficient of m' may indeed be distinctly higher than the value given by Hartridge and Roughton for that of their k_2, but further investigations are needed.

SECTION 2—THE RATE OF DISPLACEMENT OF CARBON MONOXIDE FROM COMBINATION WITH HÆMOGLOBIN BY OXYGEN

A—*Experimental*

In the case of this reaction some further experimental information is desirable.

Most of the experiments were done with the moving fluid method; very dilute solutions of carboxyhæmoglobin (1 part blood to 1000 or more parts buffer solution) were mixed with equal volumes of oxygenated water (in equilibrium with gas phases containing 1 atmosphere or more of oxygen). Such amounts of dissolved O_2 are sufficient to cause the %COHb to drop from an initial value between 90 and 100% to a final value between 30 and 40%: this gives sufficient range for observations at intermediate percentages to be made. Further details as to the preparation of the solutions are given in Part IV.

The mixed fluid passed through suitable intermediate volumes, and thence through a 20-cm observation tube of the type figured by Hartridge and Roughton (1926, fig. 2). Herein were made determinations of %COHb in the running fluid. In most tests the fluid took rather less than 1 second to traverse the observation tube; this time is very short compared with the half-period of the reaction at room temperature, so there should have been no appreciable heterogeneity error due to the reaction having proceeded further in some parts of the spectroscopic fluid under observation than in others.

The observation tube was wrapped in silver paper, so as to collect as much light as possible on to the spectroscope, which was carefully clamped, so as not to shift relatively to the tube. As source of light a 60-watt Opalite lamp was used. Controls showed that if the lamp was placed at least 20 cm from the observation tube, it did not cause any appreciable photochemical dissociation of COHb in the fluid whilst traversing the tube. This is a necessary precaution, since, as is well known, light displaces CO from combination with hæmoglobin in favour of O_2; this effect is very pronounced in dilute solution, for a given decrease in %COHb produces a correspondingly smaller increase in [CO] than in strong hæmoglobin solutions.

B—*Tests of the Equation for the Reaction*

Equation (10.2) of Part IV, if rearranged as regards signs, runs

$$-d\,[\mathrm{COHb}]/dt = m\,[\mathrm{COHb}] - \frac{m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]}{[\mathrm{O_2}]}.$$

This equation has been tested with satisfactory results in the present reaction also.

In order to get clear cut results it is obviously desirable, if possible, to use an $[O_2]$ high enough for the back reaction term, *i.e.*, $-m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]/[\mathrm{O_2}]$, to be negligible in comparison with the rate of dissociation of COHb, for under such conditions the simple equation $d\,[\mathrm{COHb}]/dt = -m\,[\mathrm{COHb}]$ should be applicable.

The easiest way to test whether our ideal has been achieved is to raise the $[O_2]$ further, and then see whether any increase in the observed rate of dissociation of COHb occurs. If no appreciable increase is noticed, then it may be concluded that in both cases the $[O_2]$ is high enough.

The test is thus exactly like that used by Hartridge and Roughton (1923, *b*) in their work on the rate of dissociation of O_2Hb in the presence of $Na_2S_2O_4$.

Here they showed that above a certain $[Na_2S_2O_4]$ the rate of dissociation of O_2Hb was not increased, and therefore that in the two stage process $O_2Hb \rightarrow O_2 + Hb$, followed by $O_2 + Na_2S_2O_4 \rightarrow$ oxidation products of $Na_2S_2O_4$, the second step occurs so much faster than the first that the overall rate of dissociation of O_2Hb is simply proportional to the $[O_2Hb]$, without any complication due to any back reaction between O_2 and Hb.

In the present paper, COHb takes the role of the O_2Hb, and O_2 takes the role of the $Na_2S_2O_4$ in the 1923 paper.

(i) *A Typical Experiment*—In fig. 2 are plotted the results of experiments at p_H 6·3 (phosphate buffer) and at p_H 10 (ammonia-ammonium chloride buffer). At each p_H two different values of $[O_2]$ in the mixed fluid were used, viz. :—

(*a*) Partial pressure of dissolved O_2 (*i.e.*, pO_2) = $\frac{1}{2}$ atmosphere.

(*b*) pO_2 = 1 atmosphere.

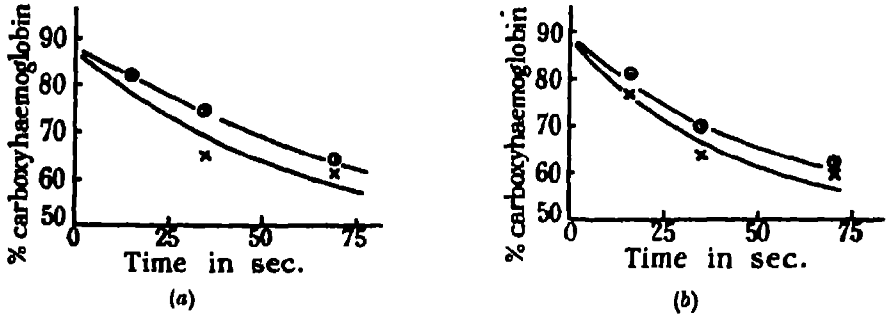

FIG. 2—Effect of varying oxygen concentration upon rate of dissociation of carboxyhæmoglobin at (*a*) p_H 6·3 and (*b*) 10·0. ⊙ $\frac{1}{2}$ atmosphere pO_2; ×, 1 atmosphere pO_2.

It will be seen that at each p_H the rate of dissociation of COHb occurs rather faster at the higher $[O_2]$ than at the lower $[O_2]$, so that our ideal has not been realized. The discrepancy between the two rates is, however, small enough for a rough extrapolation to the ideal condition to be carried out as follows.

The slower rate of dissociation of COHb, when $pO_2 = \frac{1}{2}$ atmosphere is due to the greater velocity of the back reaction between CO and Hb. According to Section I, the velocity of the back reaction $= m'$ $[CO]$ $[O_2Hb]/[O_2]$. Therefore, in fig. 2, the back reaction rate between $t = 0$ and $t = 70$ seconds should be, with sufficient accuracy, twice as great when $pO_2 = \frac{1}{2}$ atmosphere, as when $pO_2 = 1$ atmosphere, *i.e.*, the back reaction rate for $pO_2 = 1$ atmosphere =

(back reaction rate for $pO_2 = \frac{1}{2}$ atmosphere — back reaction rate for $pO_2 = 1$ atmosphere). Since for

$$\left.\begin{array}{l} pO_2 = \frac{1}{2} \text{ atmosphere [COHb]} = 62\% \\ pO_2 = 1 \text{ atmosphere [COHb]} = 59\% \end{array}\right\} \text{at 70 seconds } p_H \text{ } 6 \cdot 3.$$

It can readily be seen that in the ideal case when the $[O_2]$ becomes so large that the back reaction between CO and Hb is negligible, the [COHb] would tend to the value 59−(62−59), *i.e.*, 56%.

The true value of the velocity constant *m* should then be given by: —

$$\log_e \frac{[\text{COHb}] \text{ at } t = 0}{[\text{COHb}] \text{ at } t = 70} = m \times 70,$$

i.e.,

$$m = 0 \cdot 0065.$$

(ii) *Four Tests of the Equation* $-d\,[\text{COHb}]/dt = m\,[\text{COHb}]$—(*a*) Log [% COHb] plotted against time should give a straight line. Fig. 3 shows that this is true within the limits of experimental error. Unfortunately, however, the points all fall within a range (viz. 90–65% COHb) where the reversion spectroscope is not very accurate. The precision in the value of *m* is hence only ± 20%.

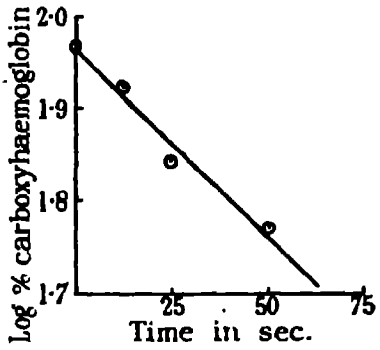

FIG. 3—Test of unimolecularity of reaction.

(*b*) The value of *m* should be independent of the initial %COHb. In one experiment at p_H 10, temperature 14° C, the initial [COHb] = 86%, [COHb] = 64% at 82 seconds—whence $m = 0 \cdot 004$. In a second experiment at the same p_H and temperature and with the same blood the initial [COHb] = 34%, [COHb] = 26% at 82 seconds—whence $m = 0 \cdot 0036$.

These two values of *m* agree with one another within experimental error in spite of the large difference in the initial %COHb.

(*c*) The rate of change of % COHb should be independent of the total Hb concentration. This occurred in two experiments of which the first was done by the present technique on blood diluted to 1 in 2000, whereas the second was done by the shaking method (described later) on the same blood diluted 1 in 50. The rate of change of %COHb was roughly the same in the two experiments.

(*d*) Comparison of the rates of the velocity constants with the equilibrium constant of the reaction. A further test of the equation proposed for the present reaction, and of that proposed for the displacement of O_2 by CO from hæmoglobin in the previous section is that the ratio of m' to m should be equal to M, the equilibrium constant of the reaction.[*]

Four such tests were made, and are summarized in Table VI. Experiment A was done on one sample of blood, and experiment B on another.

With the exception of the p_H 6·2 test in experiment B the agreement between m'/m and M is within the limits of experimental error: the discrepancy in this one test may perhaps be due to some chance error in the measurement of m or m'. If this doubtful test is excluded, the average value of the ratio m'/m to $\mathrm{M} = 1{\cdot}02$.

Table VI

	p_H	m'	m	m'/m	M	$m'/m \div \mathrm{M}$
Experiment A— Temperature 18° C ...	6·2	1·95	0·0107	182	198	0·92
	11·0	1·50	0·0083	181	221	0·82
Experiment B— Temperature 16° C	6·2	2·90 ?	0·0078	370 ?	207	1·78 ?
	10·0	2·13	0·0091	234	177	1·32

In general it may be concluded that within the rather wide limits of experimental error of the present observations, the rate of dissociation of COHb in presence of excess of O_2 does conform to the equation

$$\frac{d\,[\mathrm{COHb}]}{dt} = -m\,[\mathrm{COHb}].$$

C—*The Effect of p_H and Temperature*

(i) *The Effect of p_H*—The effect of p_H is very slight, as may be seen by comparing the values of m at p_H 6·2 and at p_H 10 in Table VI.

In experiment A the ratio of m at p_H 6·2 to m at p_H 10 = 1·29

In experiment B the ratio = 0·86.

In a third experiment the ratio = 1·14.

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[*] [*Note added in proof, June* 20, 1934—M was obtained in the following way: dilute O_2Hb solution was shaken with a series of gas mixtures containing suitable known pressures of CO and of O_2. The %COHb, when equilibrium had been reached, was measured by the reversion spectroscope. M is then given by the average value of $[O_2]\,[\mathrm{COHb}]/[\mathrm{CO}]\,[O_2\mathrm{Hb}]$.

The nearness of the average value of the ratio, viz., 1·10, to unity shows that the effect of p_H, if any, is within experimental error. The result will be discussed further in the next section.

(ii) *The Effect of Temperature upon m*—The study of this factor is not yet completed, but the results so far indicate a rather high temperature coefficient, viz., of the order of 4 per 10° C.

D—*Preliminary Experiments with the Shaking Method*

Description of Technique—One part of blood is diluted with 40–200 parts of buffer solution, and shaken with a suitable pressure of CO so as to saturate the hæmoglobin, without having an appreciable amount of CO present in physical solution.

5 cc or more of this solution is then introduced into a tonometer containing an oxygen gas mixture; the volume of the gas phase in the tonometer should be at least 100 times greater than that of the liquid phase, otherwise the CO which dissociates from the Hb and escapes into the gas phase will mount to too high a pressure. The tonometer is at once violently shaken for a known period, at the end of which the tonometer is stood vertically, and the blood solution run out as quickly as possible into a glass trough. The %COHb is then determined at leisure by the reversion spectroscope. Whilst the solution stands in the trough, there is a further slight fall in %COHb owing to the system proceeding to equilibrium in the liquid phase between the O_2, CO, and Hb in solution. Calculation, however, shows that usually this correction is not more than 2% COHb with the stronger solutions of hæmoglobin used in the present method.

Practical Application of the Method—(i) It has already been mentioned that the shaking method and the moving fluid method give concordant results over the range 90–70% COHb, wherein the correction for the back reaction is in both methods slight.

As an example of the application of the method, the following experiment may be quoted.

30 cc of CO-blood solution (1 in 40), p_H 10, temperature 14° C, were shaken in a 4-litre tonometer with a gas mixture containing O_2 at pressure of (a) 350 mm Hg, (b) 960 mm Hg. After 4 minutes shaking the percentage of COHb was in (a) 43%.

The correction for the further change in percentage of COHb whilst the solution stands in the trough is, in this test, only 0·5%. The true value of the [COHb] at the end of shaking was therefore 43·5%.

In (b) the observed figure was 37% and the final correction figure 39%. The oxygen gas phase and the shaking are, in this example, not quite sufficient to permit the dissociation of COHb to proceed unopposed. Extrapolation to the ideal condition can, however, be made in the same manner as before, the figure so obtained is $39 - (43 \cdot 5 - 39) \times 350/(960 - 350)$, *i.e.*, 35·5%, and the value of $m = 0 \cdot 0040$.

(ii) The method has also been applied in two tests to a suspension of unlaked corpuscles. No difference could be found in the rate of dissociation of COHb whether the corpuscles were laked or unlaked. Use of this observation has already been made elsewhere by the writer (Roughton, 1932) in working out the joint effects of diffusion and of chemical reaction velocity in the red blood corpuscle.

SECTION 3

A—The fact that the two reactions studied in this paper conform to the equation

$$d\,[\mathrm{COHb}]/dt = \frac{m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]}{[\mathrm{O_2}]} - m\,[\mathrm{COHb}]$$

both as regards their velocities and their equilibrium is most readily explained by the hypothesis that the complete reactions really consist of a series of pairs of reactions:

$$\left.\begin{aligned}&\mathrm{Hb_4O_8} \rightleftharpoons \mathrm{Hb_4O_6} + \mathrm{O_2}\\&\mathrm{Hb_4O_6} + \mathrm{CO} \rightleftharpoons \mathrm{Hb_4O_6(CO)}\end{aligned}\right\}$$
$$\left.\begin{aligned}&\mathrm{Hb_4O_6(CO)} \rightleftharpoons \mathrm{Hb_4O_4(CO)} + \mathrm{O_2}\\&\mathrm{Hb_4O_4(CO)} + \mathrm{CO} \rightleftharpoons \mathrm{Hb_4O_4(CO)_2}\end{aligned}\right\}$$
$$\left.\begin{aligned}&\mathrm{Hb_4O_4(CO)_2} \rightleftharpoons \mathrm{Hb_4O_2(CO)_2} + \mathrm{O_2}\\&\mathrm{Hb_4O_2(CO)_2} + \mathrm{CO} \rightleftharpoons \mathrm{Hb_4O_2(CO)_3}\end{aligned}\right\}$$
$$\left.\begin{aligned}&\mathrm{Hb_4O_2(CO)_3} \rightleftharpoons \mathrm{Hb_4(CO)_3} + \mathrm{O_2}\\&\mathrm{Hb_4(CO)_3} + \mathrm{CO} \rightleftharpoons \mathrm{Hb_4(CO)_4}\end{aligned}\right\}$$

and that the simplest Langmuir principles apply to all these constituent reactions. This idea has already been developed at the end of Part IV, to which reference should be made. In this connection one related point may be emphasized. The equilibrium between oxygen and hæmoglobin, on the general intermediate compound hypothesis, is given by the equation

$$\frac{\%\mathrm{O_2Hb}}{100} = \frac{\mathrm{K_1}p + 2\mathrm{K_1K_2}p^2 + 3\mathrm{K_1K_2K_3}p^3 + 4\mathrm{K_1K_2K_3K_4}p^4}{4\,(1 + \mathrm{K_1}p + \mathrm{K_1K_2}p^2 + \mathrm{K_1K_2K_3}p^3 + \mathrm{K_1K_2K_3K_4}p^4)},$$

where K_1, K_2, K_3, K_4 are the respective equilibrium constants of the intermediate reactions, and $p = [O_2]$. At high $[O_2]$ this equation reduces to the simplified form

$$\frac{[O_2Hb]}{[Hb][O_2]} = 4K_4,$$

i.e., on the intermediate compound hypothesis the dissociation curve should, at high values of $[O_2]$, tend to become a rectangular hyperbola, even if it is S-shaped at lower values. This consequence has never been verified by direct experiment, although indirect confirmation is given by the facts recorded in Tables II, III, and IV of Section 1, namely, that at varying high $[O_2]$, but constant [CO] and $[O_2Hb]$, the rate of displacement of O_2 by CO is over a wide range inversely proportional to $[O_2]$.

B—*The Effect of* p_H.

In both reactions the relatively slight effect of p_H is anomalous and needs further discussion.

(i) *The Effect of* p_H *on* m'—On the general view that the displacement of O_2 from combination by CO involves first of all dissociation of an O_2 molecule from hæmoglobin and then a combination of CO with the gas-group so set free, it was at first surprising to find that m' was not markedly larger in acid solutions. For the affinity of O_2 for mammalian hæmoglobin is as a rule much smaller in acid solutions, and hence there should, at given $[O_2]$, be far more free gas-groups available for CO to combine with than in alkaline solutions.

On the intermediate compound hypothesis, however, it is not essential that m' should be largely affected by p_H. For on this view

$$m' = \frac{l'_4}{4K_4},$$

where l'_4 = velocity constant of any of the reactions :—

$$\left.\begin{array}{l} CO + Hb_4O_6 \rightarrow Hb_4O_6(CO) \\ CO + Hb_4O_4(CO) \rightarrow Hb_4O_4(CO)_2 \\ CO + Hb_4O_2(CO)_2 \rightarrow Hb_4O_2(CO)_3 \\ CO + Hb_4(CO)_3 \rightarrow Hb_4(CO)_4 \end{array}\right\}$$

K_4 has already been defined, and consideration soon shows that previous data give no information as to how l'_4 and K_4 are affected by p_H.

Thus the results in Part V on the rate of combination of CO with fully reduced Hb clearly do not tell us anything about any individual velocity constant in the chain of the reactions of the intermediate compound theory. We cannot be sure that the value of l'_4 does not decrease in acid solutions and thus counterbalance the effect of any decrease in K_4 upon the value of $l'_4/4K_4$, *i.e.*, m'.

Furthermore, although the dissociation curve of O_2Hb is greatly shifted by acidity, this does not necessarily mean a large effect on K_4. For, in the general equilibrium equation of the intermediate compound theory, the terms $K_1K_2p^2$ and $K_1K_2K_3p^3$ are as a rule found to be unimportant as compared with the K_1p term and the $K_1K_2K_3K_4p^4$ term, especially the latter. As long as K_1 and $K_1K_2K_3K_4$ decrease largely with increasing acidity, the requisite effect of acid on the dissociation curve is explicable. Now a large decrease in K_1 and in $K_1K_2K_3K_4$ does not necessarily involve a large change in K_4. Thus until some independent method is available for measuring l'_4 and K_4 (for suggestions in regard to the latter see the discussion by Forbes and Roughton (1931)), we cannot on the intermediate compound theory predict, with any safety, what effect p_H should have upon m'.

There is also another possible explanation raised by some experiments given in a later paper of the present series. When an O_2 molecule dissociates from Hb_4 and is replaced by CO under the conditions of the present paper, the length of time during which the combining group of the Hb_4 molecule so concerned is free, *i.e.* not combined either with O_2 or CO, must be very short, *i.e.* of the order probably of 1/100 second, as suggested by rough calculation. It is conceivable that there may be a quick equilibrium between oxygen and hæmoglobin, which differs from the usual slow equilibrium observed in dissociation curve experiments with tonometers, owing, possibly, to some tautomeric change occurring after the quick equilibrium is reached. If this were so, then the effect of p_H upon such a quick equilibrium might well be different from its traditional effect on the oxyhæmoglobin dissociation curve. The matter will be raised again in Part VII wherein another possibility of a similar kind will be discussed.

(ii) *The Effect of p_H on m*—If m' is but little affected by p_H, and the same is also true of the equilibrium constant of the reaction, M (as has already been shown by numerous investigators and confirmed by us over the p_H range 5·6–10·0), then since $m'/m = M$, it would be expected that m should be likewise but little affected, as is shown to be the case in Section 2.

From another point of view, however, the slight effect of p_H upon m is surprising. The rates of combination both of O_2 and of CO with Hb are only slightly dependent on p_H, but the O_2-dissociation curve and the CO-dissociation curve of hæmoglobin are both markedly affected by p_H, and so also according to Hartridge and Roughton (1923, *b*) and Millikan (1933) is the rate of dissociation of O_2Hb in the presence of sodium hydrosulphite. Since the CO reactions with Hb are so closely parallel in every way to the O_2 reactions, it might well be expected that the velocity of dissociation of COHb would be greatly affected by p_H, just as is the velocity of dissociation of O_2Hb in presence of $Na_2S_2O_4$.

There is, however, an important difference between the two cases. When oxyhæmoglobin dissociates in presence of $Na_2S_2O_4$, the Hb_4 molecules, on the intermediate compound hypothesis, pass successively through the stage of having all four of their gas combining groups saturated to the zero stage in which none of these groups are saturated. But when carboxyhæmoglobin dissociates in the presence of excess of oxygen, the Hb_4 molecules would never have, in appreciable degree, less than three of their gas combining groups occupied by O_2 or CO, and for the most part would have all four of the groups occupied by one or other of these molecules. It is possible, as already suggested in discussing the relatively slight effect of p_H upon m', that the effect of p_H upon equilibria and velocity constants may only show itself, when there is present an appreciable amount of hæmoglobin with *less than three* groups occupied by O_2 or CO.

Very accurate observations on the CO-Hb dissociation curve at the extreme top (say, between 98 and 100% COHb) if carried out at different p_H's should throw light on this point as regards equilibria. Preliminary calculation has shown that the necessary accuracy might be reached if relatively large volumes of completely saturated CO-Hb solution were shaken with relatively small gas phases and the amount of CO evolved into the gas phase at equilibrium were then measured by a modification of the methods used by Forbes and Roughton (1931). It is hoped later to test this proposal.

As regards velocity constants the idea could be conceivably tested further in two ways.

(*a*) If some suitable reagent could be found, which would combine with dissolved CO, without reacting directly on the hæmoglobin, then the rate of dissociation of COHb could be observed under the same conditions as the rate of dissociation of O_2Hb in presence of $Na_2S_2O_4$ has been observed, and it would then be most interesting to see whether the effect of p_H was similar to its effect in the latter test.

Unfortunately, however, the type of reagent available for the purposes, such as that used for absorption of CO in gas analysis, is harmful to the hæmoglobin.

Some preliminary experiments at p_H 10 have been carried out on the rate of dissociation of carboxyhæmoglobin in presence of ferricyanide. According to Conant (1923) there is no direct action of ferricyanide on COHb or O_2Hb, but dissociation occurs as a result of a two stage process, viz., COHb → CO + Hb followed by Hb + ferricyanide → MetHb.

The actual rates of dissociation were observed to be about 50% faster than the rate of dissociation of COHb in presence of O_2. This method, however, can scarcely be held to be suitable for our purpose. For on the intermediate compound hypothesis, we must assume that there also exist compounds intermediate in composition between that of fully reduced hæmoglobin and that of methæmoglobin. During all but the earliest stages of dissociation of CO there may be in existence hæmoglobin molecules in which one or more of the combining groups is combined with CO, one or more of the groups is free, and one or more of the groups is turned into a methæmoglobin type of group.

There is no reason to suppose that the rate of dissociation of CO from a hæmoglobin molecule of this triple character would be the same as from a hæmoglobin molecule, with its combining groups either free or only occupied by CO.

(*b*) If, in the displacement of O_2 by CO from Hb, the concentration of CO could be raised, and that of O_2 lowered, enough for the rate of the two-stage process to be governed not by the second step, *i.e.*, the rate of combination of CO, but by the first step, *i.e.*, the rate of dissociation of O_2 from the hæmoglobin molecule, then it might be possible to find out whether the rate of dissociation of the first of the four O_2 molecules, which is combined with the hæmoglobin molecule when the latter is saturated with O_2, was insensitive to p_H just as is apparently the rate of dissociation of the first CO molecule. A preliminary experiment of this kind was tried, and only slight difference was observed in the rate of decay of the percentage of oxyhæmoglobin at p_H 6·0 and 10·0. Unfortunately, however, the concentrations of CO and of O_2 could not with the apparatus available be so regulated as to ensure that the first step of the two-stage process was the sole governor of the rate of the whole process. It is hoped to make some further attempts along this line.

One further point may be mentioned in conclusion, still assuming the applicability of the intermediate compound hypothesis. If the first of the four CO molecules combined in 100% carboxyhæmoglobin dissociates at a rate almost independent of p_H, whereas the complete dissociation of all four CO molecules (when carried out in a manner analogous to the dissociation of oxyhæmoglobin in the presence of $Na_2S_2O_4$) dissociates much faster in acid than in alkaline solution, then the only remaining explanation would seem to be that some or all of the CO molecules, which dissociate after the first one, must do so much more quickly in acid p_H than in alkaline solution.

SUMMARY

1—The velocity with which CO displaces O_2 from combination with hæmoglobin is expressible by the equation:

$$d\,[\mathrm{COHb}]/dt = \frac{m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]}{[\mathrm{O_2}]}$$

over the first half of the reaction, during which the velocity of the back reaction is negligible. The equation was tested with a wide range of concentrations of each reagent and found to be valid. m' is but slightly affected by p_H, and the quantitative results accord with the intermediate compound hypothesis of Adair, rather than with Hill's equation.

2—The velocity with which O_2 displaces CO from combination with hæmoglobin conforms to the equation

$$-\,d\,[\mathrm{COHb}]/dt = m\,[\mathrm{COHb}] - \frac{m'\,[\mathrm{CO}]\,[\mathrm{O_2Hb}]}{[\mathrm{O_2}]}.$$

The value of m can be calculated from experiments at two different values of $[\mathrm{O_2}]$.

It is found that, within experimental error, $m'/m = \mathrm{M}$, where $\mathrm{M} =$ the equilibrium constant of the reaction $\mathrm{CO} + \mathrm{O_2Hb} \rightleftharpoons \mathrm{O_2} + \mathrm{COHb}$. m also is but slightly affected by p_H.

3—The relatively slight effect of p_H both on m, and on m', was rather unexpected, but its theoretical implications are discussed at length.

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APPENDIX

COMPARISON OF RESULTS OBTAINED BY SEVERAL INDEPENDENT METHODS FOR MEASURING THE RATE OF DISPLACEMENT OF OXYGEN FROM COMBINATION WITH HÆMOGLOBIN BY CARBON MONOXIDE

A—*The " Light " Method and the " Mixing " Method*

Hartridge and Roughton's earliest work on this reaction (1923, *a*) antedated their invention and application of the mixing-chamber technique. Instead, they irradiated a dilute solution of COHb (containing also much dissolved O_2) with light of strong intensity, thus causing the combined CO to be replaced by O_2. In the dark the system returned to its original condition and observations on the rate of return were made by—

(i) *A flow method*, in which the solution after passing through a brightly lit tube emerged into a darkened tube, at various points of which the %COHb was determined in the running fluid.

(ii) *A stationary method.* In this the irradiating light intensity was suddenly much reduced, and observations were made on the rate of shift of the α-absorption band in the spectrum as the system returned to the COHb condition. Fuller details of the method are recapitulated below.

The numerical results of the present paper by the mixing methods agree well with those obtained by the original " light " method ; to clinch the matter it is necessary to compare the results, by the two methods, on hæmoglobin prepared from the same blood and with identical values of [CO], [O_2], and [O_2Hb]. The details of this test were as follows : bottle A contained a solution of CO, and bottle B a solution of Hb prepared in the usual way, the concentrations of the reagents being so adjusted that the time required for the resultant reaction to proceed to half completion was about 5 seconds. The two bottles were then connected with a mixing apparatus having an observation tube of 12·8 mm diameter. The spectroscope was then set at a very short distance from the mixing chamber with the micrometer screw so adjusted that the α-bands of a solution of 70% COHb would be in exact alignment, and the taps of the two bottles turned on. Whilst the solution was running the percentage of COHb was practically nil, and the α-bands in the two spectra therefore appeared quite out of alignment. The two taps were then suddenly turned off at the same instant. As the reaction proceeded in the stopped fluid the two α-bands moved relatively to one another till finally at an instant reported by the observer they appeared in alignment with one another. The time taken from the instant at which the taps were turned off to the instant of correct alignment was noted by means of a stop-watch. The following times were obtained in a series of readings in one experiment: 7·4, 8·6, 8·2, 9·2, 8·2, 10·0, 10·6, 8·0, 8·6, 8·2, 9·2 seconds, average = 8·8 seconds.

The intensity of light passing through the solution was now increased 100-fold by means of the technique described in an earlier paper (Hartridge and Roughton, 1925), the solution in the observation tube being still that remaining at the end of the above experiment. The strong light intensity caused the whole of the CO to be displaced from the Hb, and O_2Hb to be re-formed. At a suitable instant the intensity of the light passing through the solution was suddenly reduced 100-fold (*i.e.*, to the same value as prevailed during the movement of the bands in the former experiment), thus causing the displacement of O_2 from Hb by CO to occur once more. The time taken by the bands to come into alignment was measured as before. The readings obtained were 8·8, 7·9, 6·8, 7·2, 11·0, 7·8, 7, 8·8, 7·8, 9·2 seconds, average = 8·3 seconds. The agreement with the previous figure, 8·8 seconds, was as good as could be expected. A similar comparison of the time taken by the solution to reach 55% COHb gave 4·0 seconds by the mixing method and 4·6 seconds by the light method.

The agreement between the two methods is not only satisfactory from the technical point of view, but also serves to answer a possible theoretical objection to the "light" method. In the latter it is assumed that the chemical effects of the strong light disappear at the moment at which the light is interrupted, and that there are no photochemical "after-effects" such as are known to occur in certain reactions. For this assumption, there was at the time of the earlier paper no independent evidence. The result of the present test, however, shows that such photochemical "after-effects," if they exist, are negligible in their effect as regards the velocity of the reaction under examination.

B—*Comparison of Results obtained by the "Flow" Method and the "Stationary" Method*

In our earlier paper (Hartridge and Roughton, 1923, *a*) we compared the results obtained by the "stationary" method with the results obtained by the

" flow " method, which has since become a basal feature in the measurement of rapid reactions. The hæmoglobin, however, was not prepared from the same sample of blood in the two tests. We therefore thought it desirable to repeat the comparison with identical conditions in each experiment. It was found that the time required to reach 25% COHb when mixing was followed by the usual flow method was 1·7 seconds, whereas when mixing was followed by the stationary method just described, the average time was 1·8 seconds. This agreement is within the limits of experimental error and so confirms the reliability of both methods.

C—Comparison of Flow Method and Shaking Method

In the flow method—

Bottle A = blood 1 in 1000, p_H 10, equilibrated with gas mixture containing 760 mm Hg O_2 pressure.

Bottle B = water equilibrated with gas mixture containing 15 mm CO 160 mm O_2.

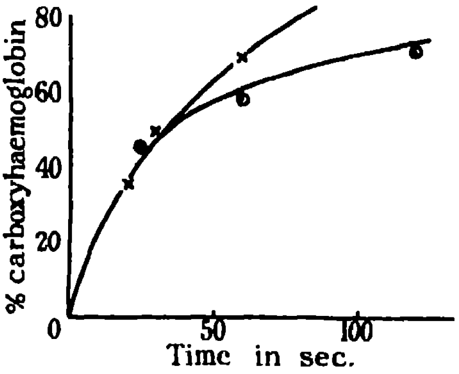

FIG. 4.—Comparison of flow method, ⊙, and shaking method, ×.

In the shaking method—

5 cc of blood solution, p_H 10, was shaken in a tonometer with a gas mixture containing 7·6 mm CO, 450 mm O_2. The result is shown in fig. 4. During the first half of each process, in which the concentrations of dissolved [CO] and dissolved [O_2] are about the same, the two methods check satisfactorily; during the latter half the shaking method gives higher results, but this is due to the fact that the final equilibrium percentage of COHb was about 10%

higher in the shaking experiment than in the flow experiment, which latter was, consequently, slowed by the back reaction at an earlier stage.

It may thus be concluded that all these varied methods give concordant results for the particular reaction in question. This confirms our confidence in the general validity of the experimental technique used in this and in other papers of this series.

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The Kinetics of Hæmoglobin VII—*Some Notes on the Reactivity of Freshly Reduced Hæmoglobin*

By F. J. W. Roughton

(Communicated by H. Hartridge, F.R.S.—Received February 2, 1934—Revised April 17, 1934)

INTRODUCTION

Various results, which are anomalous from the theoretical point of view, have been described and discussed in our previous papers, notably in those dealing with the displacement of O_2 by CO from combination with hæmoglobin, and *vice versa*. The fact that these anomalies were specially found where dissociation of 1 molecule, *e.g.*, of O_2 from hæmoglobin, is quickly followed by combination with another molecule (*e.g.*, of CO), so that the group in the hæmoglobin molecule concerned only remains free for a very short time, suggested to us the possibility that such freshly formed and short lived groups might differ in reactivity from that of the same groups, when allowed a longer period of life before meeting the second reactant.

For the sake of brevity such freshly-formed and shortly-lived molecules will be referred to as " new " molecules, whilst the molecules with which they are to be contrasted in activity will be called " old."

Hartridge and Roughton (1926) have already pointed out that by using a number of their mixing chambers in series, the reactivity of transient intermediate compounds can be detected and followed. Such technique, which will be described more fully below, has been applied to several types of hæmoglobin reaction, and though in most experiments the results have been negative, there were two reactions in which the results were consistently positive:

(*a*) The rate of combination of CO with " new," as compared with " old " reduced Hb at alkaline p_H and room temperature.

(*b*) The equilibrium between O_2 and " new," as compared with " old " Hb at acid p_H.

SECTION I—RESULTS

A—*The Reactivity of* CO *with " New " and " Old " Hæmoglobin*

The experimental arrangement is shown in fig. 1, four large stoneware bottles A, B, C, D were half filled with the following solutions :—

A—Water free of dissolved O_2, to which had been added completely reduced blood (1 part blood to 15–25 parts water), *i.e.*, a dilute solution of completely reduced blood.

B—Aerated water + the same proportion of the reduced blood used in A, the blood, however, being reoxygenated before being mixed with the water.

C—0·4% $Na_2S_2O_4$ solution made up in ammonia-ammonium chloride buffer p_H 10, or phosphate buffer p_H 6·6.

D—Water containing a suitable amount of dissolved CO.

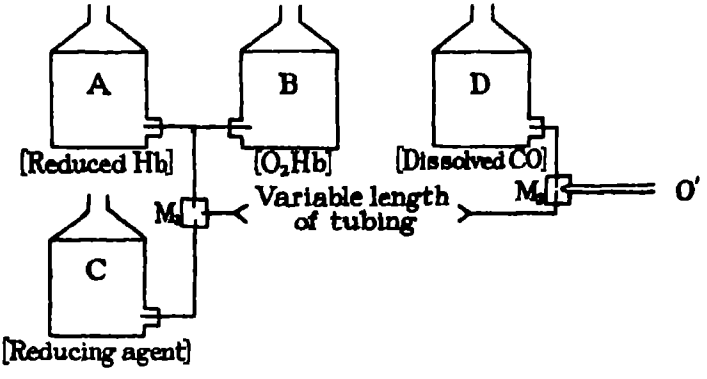

FIG. 1—Arrangement for measuring rate of combination of CO with " old " and " new " reduced hæmoglobin.

The leads from A and B were joined by a T-piece, the stem of which connected with one inlet of a mixing chamber M_1.

The second inlet of M_1 was joined to C, whilst the outlet of M_1 was connected by a variable length of tubing to the inlet of a second mixing chamber M_2. The other inlet of M_2 was joined to D. By applying compressed gas to A, B, C, D, and opening the appropriate clips on the leads issuing from them, either the combination A + C + D, or B + C + D was caused to run, and the rate of formation of COHb determined in the usual way by spectroscopic observation upon the fluid issuing from M_2 into the observation tube O′ attached thereto. With the combination A + C + D, the rate of the reaction CO + "old" Hb is measured, whereas with the combination B + C + D the rate of the reaction CO + "new" Hb is measured, the "new" Hb being that which is freshly formed by dissociation of the O_2Hb. The volume of tubing connecting M_1 to M_2 was adjusted so that there was just time for the O_2Hb to be almost completely reduced before reaching M_2. At room temperature the time allowed between M_1 and M_2 was usually about 0·2 second, but in some experiments this was shortened to 0·1 second.

An alternative method of doing the "old" Hb experiment is to dispense with the bottle A altogether, and to insert a by-pass of much larger volume between M_1 and M_2; *i.e.*, so that the "time" between M_1 and M_2, if this route is taken, is at least 2 seconds. In practice experiments were carried out by both methods.

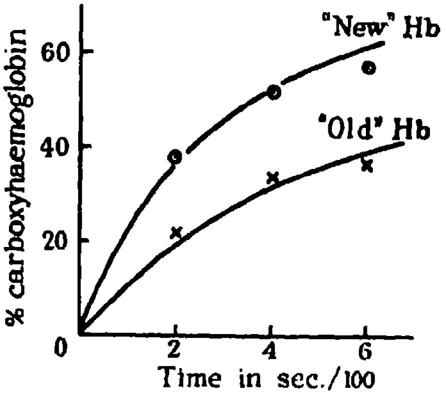

FIG. 2—Difference between rate of combination of CO with "new" and "old" reduced hæmoglobin at p_H 10.

Where possible, it was arranged that the observer, who took the spectroscopic readings on the fluid moving down the observation tube was ignorant of whether he was examining the ACD combination or the BCD combination, and his actual readings were taken down for him without his knowing what they were.

The results of one such experiment at p_H 10, room temperature *circa.* 15° C is shown in fig. 2. The difference between the reaction velocities is well outside experimental error, the ratio of the velocity constant for the CO + "new" Hb reaction to the velocity constant for CO + "old" Hb reaction being about 2. The final per cent COHb reached after several seconds was, however, the same in the two experiments.

Four similar experiments on other samples of blood at the same p_H and temperature gave values of the ratio of 2, 1·7, 2·9, 2·5, whence the average value = 2·2.

At p_H 10 and 33° C, however, the two reaction velocities agreed with one another within experimental error.

Two experiments at p_H 6·6 and at room temperature gave values for the ratio of 1·0 and 1·2, mean = 1·1. This discrepancy is not outside the limits of experimental error.

As mentioned already, the time, during which the $Na_2S_2O_4$ was allowed to act upon the oxyhæmoglobin before the mixing with the dissolved CO occurred, was arranged to be sufficient for practically complete dissociation of the O_2Hb. If, however, the dissociation was not quite complete, this should tend to retard rather than to hasten the subsequent reaction with CO. It does not therefore seem possible to explain the faster rate of the CO + " new " Hb reaction in this way.

It might also be held that the $Na_2S_2O_4$ is in some way responsible for the effect. Against this, however, may be quoted a control experiment in which the concentration of the $Na_2S_2O_4$ was varied 4-fold. There was no change in the velocity of the CO + " new " Hb reaction. A second objection is to be found in the p_H 10, 35° C experiment. It is hard to see why a rise in temperature should abolish an effect due to $Na_2S_2O_4$.

Our present view is that these experiments can only be explained by the occurrence of some relatively slow change in the hæmoglobin molecule after reduction.* Whether this change is of a tautomeric type, or of a more physical type, *e.g.*, de-activation of activated molecules, is at present obscure. But further confirmation is needed, particularly with alternative technique to the reversion spectroscope for following the progress of the reaction.

We may now summarize our negative experiments, since these were all of the type just mentioned.

(i) It was of great interest to see whether " new " Hb prepared as above differed from " old " Hb in its reactivity with O_2 as well as with CO.

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* [*Note added in proof, June* 20, 1934.—It is also possible that the sudden dilution of the hæmoglobin which occurs in these experiments may alter the molecule in some way, and thus be in part responsible for the observed effect. This possibility has been suggested to us by the anomalous behaviour of the diffusion constant of hæmoglobin on dilution as reported recently by Tiselius and Gross (1934). We hope to investigate this aspect in further detail.]

No difference could be found either at p_H 6·6 or at p_H 10 at room temperature.

(ii) Reduced hæmoglobin solution was oxygenated by mixture with aerated water in mixing chamber M_1. Thence after a variable intermediate length of tubing, it flowed into mixing chamber M_2, where is met a solution of $Na_2S_2O_4$.

The rate of dissociation of O_2Hb in the mixed fluid emerging from M_2 was measured in the usual way. No difference could be found in the rate of dissociation of " new " oxyhæmoglobin and of " old " oxyhæmoglobin either at p_H 6·6 or at p_H 10, at room temperature.

(iii) Three mixing chambers were used in series. The fluids entering the first two mixing chambers were identical with those entering the two mixing chambers in experiment (ii) so that the fluid emerging from the second mixing chamber and thence entering the third mixing chamber was reduced hæmoglobin formed either from " new " or from " old " oxyhæmoglobin according to the length of tubing between the first and second mixing chambers.

The other fluid entering the third mixing chamber was a solution of CO in water. As a result it was found that the velocity of the reaction CO + " new " Hb (formed from " new " O_2Hb) was the same as the velocity of the reaction CO + " new " Hb (formed from " old " O_2Hb), both at p_H 6·6 and at p_H 10, room temperature.

For this blood solution a control confirmed that the velocity of the CO + " new " Hb reaction at p_H 10 was twice as great as the velocity of the CO + " old " Hb reaction.

The peculiar condition of the " new " hæmoglobin molecule does not therefore seem to depend upon the age of the oxyhæmoglobin from which it is formed provided this age is greater than 0·1 second. We have not data for oxyhæmoglobin of shorter life.

B—*The Quick and Slow Equilibrium between Oxygen and Hæmoglobin and the Effect of* p_H *thereon*

For these observations the following experimental arrangement was adopted, fig. 3.

A 5-litre stoneware bottle P contained 3·5 litres of O_2-free water. To this was added 1·5 cc oxygenated sheep's blood; the fluid when well mixed thus consisted of a dilute solution of blood containing 80–90% O_2Hb.

The lead from P was joined to one side of a glass mixing chamber M_1 whilst the other inlet of M connected to a manifold to which were joined leads from three or more stoneware bottles Q, R, S, etc. Q, R, S, etc., contained O_2-free buffer solutions of p_H ranging from 10·0 to 5·6.

The outlet of M was connected through a dead space volume of about 6 cc with the 20-cm observation tube used in our experiments on the rate of dissociation of COHb (*see* Part VI).

The solution from P and from one of the bottles, Q, R, or S, was driven by compressed nitrogen through the mixing chamber and observation tube, and readings of the %O_2Hb in the latter were taken by the reversion spectroscope, both on the running fluid and on the stationary fluid obtained by clipping off the two bottles P and Q (or R or S, etc., as the case may be) *suddenly* and *simultaneously*.

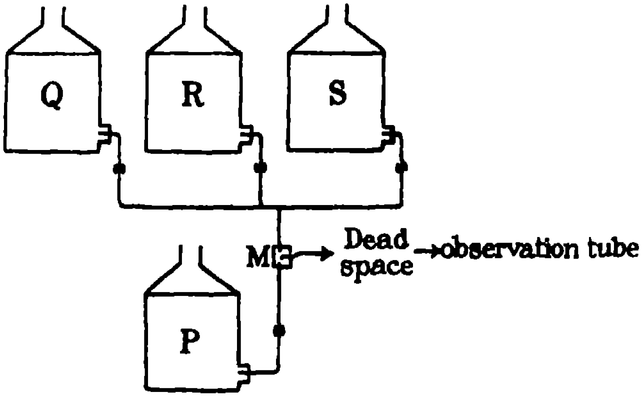

FIG. 3—Arrangement for studying slow and quick equilibria of oxygen with hæmoglobin.

The mixing of the fluid from P with an equal volume of O_2-free solution leads to a decrease in the percentage of O_2Hb,* The readings on the running fluid, which corresponded to a time of 0·5 to 0·7 seconds from mixture, should indicate the position of the quick equilibrium; for, according to the experiments of Hartridge and Roughton (1923) on the rate of dissociation of sheep oxyhæmoglobin, a time of 0·5–0·7 seconds ought to be sufficient for an equilibrium to be reached. The readings on the stationary fluid, however, should give the final value of the "slow" equilibrium, if spectrocopic readings are repeated over a period of several minutes till constant values are obtained.

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* The more dilute the solution of hæmoglobin, the larger the change in percentage of O_2Hb to be expected. Hence the present technique was designed for the most dilute Hb solutions feasible.

The results of these experiments which were all conducted at or near 15° C were as follows:—

(i) At p_H 10 no difference in the quick and slow equilibrium could be detected. This experiment shows that even at p_H 10 ample time had been allowed for oxyhæmoglobin dissociation, of the type studied by Hartridge and Roughton (1923), to occur. At p_H 6·3 where the type of dissociation is much quicker there should be no doubt of enough time having been available, and any further dissociation would have to be attributed to some other process.

(ii) At p_H 6·3 (phosphate buffer) the "quick" equilibrium value was 45% O_2Hb whilst the "slow" equilibrium value was less than 20% O_2Hb and indeed impossible to measure accurately with the spectroscope.

At p_H 6·3 (acetate buffer) essentially similar results were found:

Quick equilibrium value = 57% O_2Hb

"Slow" equilibrium value = 20% O_2Hb.

At p_H's more acid than 7, there is a danger of inactivation of the hæmoglobin, especially in dilute solution. The possibility that the difference between the "slow equilibrium" value and the "quick equilibrium" value may be due to an inactivation of the hæmoglobin having occurred in the "slow" reaction, was tested by collecting some of the fluid which had passed through the apparatus, and after allowing it to stand, reoxygenating it. No difference was found between the spectroscopic reading of this oxygenated fluid, and the reading for oxygenated blood solution of the same strength, which had not been made acid, but instead had been kept at p_H 10, where there is marked stability against inactivation. Hence it is hard to see how any irreversible inactivation could have taken place.

Evidence of inactivation, however, was found by this test when acetate buffers of p_H 5·0 were used, and also with acetate buffer solutions of p_H 6·3 (but not with phosphate buffer solutions p_H 6·3) when a brass metal mixing chamber was used in place of a glass one. In the latter experiment the inactivation might be attributed to traces of copper acetate formed by passage of the fluid through the brass mixing chamber, and the subsequent action of these traces upon the dilute hæmoglobin solution. Since copper phosphate is very insoluble this effect would be less likely to arise with phosphate buffers.

(iii) The difference between the "quick" equilibrium values at p_H 6·3 and at p_H 10 respectively was only very slight, *i.e.*, of the order of 5% O_2Hb, since most of the "quick" equilibrium values lay within the range 50–60% COHb whatever the p_H value.

The difference between the "slow" equilibrium values at p_H 6·3 and at p_H 10 respectively was at least 40% O_2Hb.

SECTION II—DISCUSSION OF THE RESULTS

The positive results described in this paper are certainly very remarkable, and it is most necessary that they should be confirmed and investigated under as wide a variety of conditions as possible, and with alternative methods of observation of the percentage of O_2Hb, even though the effects recorded are so large as to be unmistakable with the reversion spectroscope. It is possible that the photoelectric methods, developed by Millikan (1933), may serve for this purpose. Until this has been done, it would be premature to engage in detailed discussion.

Three issues need only be pointed out at this stage :—

(i) The two positive effects found above are both in the right direction to contribute to the explanation of an anomaly commented on in Part VI, namely, that the rate at which CO displaces O_2 from combination at p_H 10 differs so little from the rate in acid solutions.

(ii) These results if established make it hard to believe that any final theory of the oxygen-carbon monoxide-hæmoglobin reactions can ever be reached from the intensive study of equilibria alone : a contribution from the kinetic side would seem to be an indispensable addition.

(iii) There is much analogy between the reactions of O_2 and CO with hæmoglobin on the one hand, and the reactions of enzymes with their substrates and inhibitors on the other hand. In the latter similar phenomena to those described in this paper would introduce a new development into enzyme kinetics.

SUMMARY

1—Previous results suggested that the reactivity of freshly formed hæmoglobin compounds might sometimes differ from their reactivity after standing. Of a number of reactions investigated, two proved positive.

2—The rate of combination of carbon monoxide with hæmoglobin freshly reduced from oxyhæmoglobin was measured by mixing the latter with $Na_2S_2O_4$ solution, and then 0·1–0·2 seconds later mixing the first mixture with a third solution containing dissolved CO. It was thus necessary to use two or more mixing chambers of the Hartridge-Roughton apparatus in series.

At p_H 10 and at room temperature the rate of the reaction with "new" Hb was about twice as fast as with Hb more than 2 seconds old. The effect dis-

appeared when the temperature was raised to 33° C, or the p_H lowered to 6·6. Controls suggested that the effect was not caused by the $Na_2S_2O_4$ present.

3—"Quick" equilibrium between oxygen and hæmoglobin was studied by driving a dilute solution of the latter into a mixing chamber where it met an equal volume of water, free of dissolved oxygen. The emerging fluid was examined after varying intervals of 0·5 seconds and upwards by the reversion spectroscope.

Both at p_H 10 and p_H about 6·3 the percentage O_2Hb dropped from about 90% to a value between 50–60% after 0·5 seconds, there being little difference between the "quick" equilibria in the two cases. At p_H 10 there was no further change on standing, but at p_H 6·3 there was often a further drop to below 20% O_2Hb. This was not due to inactivation of the hæmoglobin.

This result, if confirmed, would suggest that the effect of acid on the affinity of oxygen for hæmoglobin may in part be due to some relatively slow secondary process.

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The Projection of the Forebrain on the Pons and Cerebellum

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[Plates 21-28]

Introduction

Although the existence of the cortico-pontine fibres has long been recognized and their functions partially elucidated in more recent times, there has as yet been no solution of the problem of their distribution in the pons and projection on the cerebellum. The thorough investigation of the problem requires prolonged and tedious experimental work and has, probably on that account, hitherto been largely neglected. But experiments are not performed only by man. In obscure recesses of the animal kindgom lie hidden experiments of Nature which, confined within the limits of the direction of evolution chosen for them, have been condemned to carry their eccentricity to its logical conclusion. Experiments of an even more casual nature have been performed by the indiscriminate invasion of every part of the human brain by pathological processes; but it is only by the patient collection and examination of vast masses of records that any continuous narrative can be extracted from the chaotic whole.

Of the three types of experiment available it is more economical of time and material to chose that which comes ready-made: the method of comparative neurology. This, even if it does not supply a complete answer, at least provides valuable clues for the more accurate direction of the other methods.

This investigation lies primarily within the realms of comparative neurology, and its starting point is with the most extravagant of all Nature's experiments in the mammalian line: the Monotremes. By a combination of macroscopic and microscopic examination, and by comparison with a large series of brains of mammals which may be regarded as representatives of the more usual trend of evolution, a partial solution of the problem under investigation has been obtained. In addition, as much experimental and clinical evidence bearing upon the matter in hand as could be obtained has been incorporated in the general conclusions. It cannot be pretended that the results of this enquiry supply a complete answer to the question of the projection of the forebrain on the pons and cerebellum, but it is felt that sufficient evidence has been collected to support very strongly the conclusions which have been reached.

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During the progress of the work some difficulty was found in adapting the results to the generally accepted cerebellar nomenclature and it was found necessary to alter this to a certain extent. Elliot Smith (1903, *b*, *c*, *d*) divided the cerebellum into three primary lobes—anterior, middle, and posterior—using the primary and secondary fissures as boundary lines. Shortly afterwards Bolk (1906) divided the cerebellum into anterior and posterior lobes only by means of the fissura prima. In his extensive investigation Ingvar (1918) supported the subdivision into three lobes, but regarded the fissura præpyramidalis as the boundary between the middle and posterior lobes. The essential difference between the classifications of Elliot Smith and Ingvar lies in the fact that the former author considered the pyramid part of the lobus medius, the latter part of the lobus posterius. More recently Riley (1930) has reverted to the division of the cerebellum into anterior and posterior lobes separated by the fissura prima. In the present investigation it has been found necessary to adopt the primary subdivisions of Bolk and Riley. Thus the cerebellum is regarded in the present paper as being composed of two lobes only—anterior and posterior—separated by the fissura prima. This practice has several points of merit which may be briefly summarized :—

1—The fissura prima is the first dividing line to appear in the cerebellum both embryologically and phylogenetically, it is the most constant of all the fissures which appear and it is the only one which completely divides the whole cerebellum from one lateral border to the other. On the other hand, the secondary and præpyramidal fissures appear much later and are not concerned in the *primary* subdivision of the cerebellum. Further, they are not constant in their order of appearance and frequently fail to subdivide the whole bulk of the organ.

2—The adoption of this simpler classification at once abolishes any essential disagreement between the subdivisions of Elliot Smith and Ingvar. The examination of a large series of cerebella has shown that, in terms of their nomenclature, both authors were correct, for in some animals the pyramid is derived from what has been called the lobus medius and in others it is just as clearly an offshoot of the lobus posterius. It is much more convenient to combine the middle and posterior lobes as one large posterior lobe : then the pyramid is merely a portion of the posterior lobe and not of any particular part of it.

3—Experimental results show that the cerebellum should be regarded as composed of two physiological units—which correspond to the anterior and posterior lobes of this paper—separated by the fissura prima. MacNalty and Horsley (1909) found that the ventral spino-cerebellar fibres are distributed chiefly posterior to the fissura prima, the dorsal spino-cerebellar fibres chiefly anterior to it. Moreover, Mussen (1927) showed that secondary fibres from the gracile and cuneate nuclei follow two pathways which accompany the dorsal and ventral spino-cerebellar fibres and are distributed in the cerebellum in exactly the same fashion. In this paper it will be shown that the cortical connections, too, are distributed in the anterior and posterior lobes symmetrically about the fissura prima.

These points have been discussed with Professor Elliot Smith who was very ready to admit their force, and it is with his full approval that subdivision of the cerebellum into anterior and posterior lobes only, separated by the fissura prima, has been adopted.

One further note of explanation is necessary. It has been found that the cortico-cerebellar connections are related chiefly, if not entirely, to the culmen of the anterior lobe and its lateral extension in the hemisphere—the anterior crescentic lobule; and to the declive, folium, and tuber of the posterior lobe and their lateral extensions—the posterior crescentic lobule, the superior and inferior semilunar lobules, the biventral lobule, and the tonsil. In this description the term "culmen" implies the culmen plus its lateral extension, the term "declive-tuber complex" the declive, folium, and tuber and their lateral extensions. The culmen in this sense means the whole of the cortical projection area in the lobus anterius and is the equivalent of the declive-tuber complex which represents the whole of the cortical projection area in the lobus posterius. As a further simplification the temporo-pontine, parieto-pontine, and occipito-pontine fibres are all classified together as "temporo-pontine" fibres.

Material and Methods

The description of *Echidna* is based upon the examination of 10 brains in all. Of these three were fully dissected and another split in median sagittal section. For microscopic examination six series of sections cut either transversely or sagittally, and stained by various methods, were available. In the case of *Ornithorhynchus* I have been compelled to rely largely upon the published descriptions by Ziehen (1897), Elliot Smith (1899, *a*, 1902), and Hines (1929).

but I have been able to examine several series of sections most of which were incomplete.

Gross marsupial material was abundant and I had access to six brains which included those of *Dasyurus viverrinus*, *Perameles nasuta*, *Macropus eugenii*, *M. thetidis*, and another macropod of unknown species, and *Phascolomys ursinus*. No complete series of any marsupial brain stained for fibres could be obtained, but I was able to examine some incomplete series of the brain of *Notoryctes typhlops*, of a wallaby and of *Dasyurus viverrinus*. Six mouse brains—of which two were dissected—were studied and also two complete series of transverse sections stained by the methods of Cajal and Ranson. In addition to the above many brains of rodents, insectivores, edentates, carnivores, ungulates, lemurs, and monkeys were used for comparison and sometimes dissected. The enquiry was completed by the examination of six human brain-stems and cerebella, and many series of transverse microscopic sections.

The specimens examined were closely compared with published accounts of similar brains. The papers by Elliot Smith (1903, *b*, *c*, *d*), Bolk (1906), and Ingvar (1918) were of great assistance as regards the cerebellum itself, and Elliot Smith's catalogue (1902) and his papers on the Edentates (1899, *b*) and Lemurs (1903, *a*) were continually consulted for information concerning brains which were not available. From all these it was possible to compare, not only the brains actually at hand, but also those which could not be obtained, and thus extend the scope of the enquiry considerably.

Thus the results depend upon ample data. It is unnecessary to describe in detail the whole of the material examined, and six brains only are employed for description. They illustrate the salient points of the account and cover adequately the whole of the field of enquiry.

The Projection of the Pons on the Cerebellum

If the point of entrance of the trigeminal nerve be taken as a rough guide, the fully-developed pons in the highest primates and man may be regarded as comprising pre- and retro-trigeminal portions, fig. 6, Plate 26. As is well known, the trigeminal nerves in the Monotremes enter the brain-stem in front of the pons, fig. 1, Plate 21. It has been shown in a previous paper (1934) that the pons in the Monotremes represents the retrotrigeminal part only of the fully-developed pons, and that the rostrum (of Elliot Smith, 1899, *a*) is the aborted rudiment of the pretrigeminal pontine development. The tip of the rostrum covers the caudal pole of the interpeduncular ganglion as does the pretrigeminal pons of other mammals and the caudal border of the pons covers the rudimentary corpus trapezoideum almost entirely, so that the sixth, seventh, and eighth nerves are attached to the brain-stem immediately against the pons.

In 1902 Elliot Smith showed that the lobus anterius of the cerebellum is enormously hypertrophied so that it occupies almost the whole of the dorsal surface as well as the anterior surface. I have shown that this hypertrophy is restricted to the culmen, fig. 1, Plate 21, for the lingula and lobulus centralis are almost normal in size. At the same time the lobus posterius is very small. The nodule, uvula, and pyramid are not diminished so the lack of development is exhibited by the declive-tuber complex. This is particularly well seen from the dorsal aspect. In the previous paper the abortion of the pretrigeminal pons has been correlated with the rudimentary condition of the declive-tuber complex; the purely retrotrigeminal pontine development with the hypertrophy of the culmen.

In practically all marsupials, and in many primitive true mammals, the pons lies entirely anterior to the entering trigeminal nerve; that is, it presents a purely pretrigeminal development, figs. 2 and 3, Plates 22, 23. In the wide interval between the pons and corpus trapezoideum the commencement of the lateral lemniscus is plainly visible. Since the corpus trapezoideum lies naked on the ventral surface of the medulla oblongata, the sixth, seventh. and eighth nerves are attached freely to its surface, well caudal to the posterior border of the pons. In the mouse the gap between pons and corpus trapezoideum is not as large as in the marsupial, but the pons is still wholly pretrigeminal, fig. 3, Plate 23.

Of the marsupials, only the blind marsupial mole, *Notoryctes typhlops*—as far as I can discover—betrays any retrotrigeminal pontine development in addition to the pretrigeminal pons. Many primitive true mammals, however, show what may be regarded as a precocious retrotrigeminal pontine development. Notable amongst these are the hedgehog, mole, agouti, and some of the edentates (especially *Choloepus* and *Orycteropus*). These will be considered when the functions of the cortico-pontine fibres are discussed.

The cerebellum in the marsupials and primitive true mammals is developed quite otherwise than in the Monotremes for it is the culmen which is rudimentary and the declive-tuber complex which is hypertrophied, figs. 2 and 3, Plates 22, 23. Thus in these animals with the reversal of development in the pons there is a reversal of development in the cerebellum, and it is impossible to avoid the conclusion that these eccentricities are causally related. It seems certain, then, that the retrotrigeminal pons is related to the culmen of the lobus anterius of the cerebellum; the pretrigeminal pons to the declive-tuber complex of the lobus posterius.

In an ascending phylogenetic scale the pons progressively enlarges in company with the cerebellum. The growing pons soon extends caudal to the entering trigeminal nerve so that a small but definite retrotrigeminal pons appears. This new growth covers the anterior border of the corpus trapezoideum. Such an extension is illustrated in the brain of the golden agouti, fig. 4, Plate 24, in whom it may be regarded as rather precocious, but is characteristic of most of the higher non-primate mammals, and is best seen in the carnivores. The non-primates appear to be unable to advance beyond this stage, and the corpus trapezoideum is always only partly covered. It will be noted that the cerebellum has increased in size, the increase affecting both the culmen and the declive-tuber complex.

It is only in the evolution of the primates that the pons attains its fullest development. *Tarsius* and the lemurs have all acquired some retrotrigeminal pontine development and this is steadily increased in the monkeys. Fig. 5, Plate 25, was drawn from the brain of a green monkey, *Cercopithecus sabæus*, in whom the pons is covering a considerable portion of the corpus trapezoideum and is beginning to encroach upon the points of attachment of the sixth, seventh, and eighth nerves. At the same time the cerebellum has enlarged enormously in the culmen and the declive-tuber complex. The growth of the culmen is revealed in the active pushing back of the fissura prima against the opposition of the expanding declive-tuber complex, so that the enlargement of this has taken place laterally rather than antero-posteriorly. The lateral expansion of the declive-tuber complex has pushed the flocculus and paraflocculus ventrally and is responsible for most of the bulk of the cerebellar hemispheres. The lobus anterius has also expanded to take part in the growth of the hemisphere. In marsupials and primitive true mammals the anterior tip of the culmen can just be seen from the dorsal aspect peeping out from under the hood-like lobus posterius. In many marsupials and mammals it is so small that it cannot be seen from the dorsal aspect. But its increasing size in company with the growth of the retrotrigeminal pons pushes the lobus posterius posteriorly and laterally and provides an expanding contribution to the cerebellar hemispheres. In the monkey the lobus anterius occupies about one-half of the median sagittal section, and the culmen is complexly divided, but it never overtakes the start of the lobus posterius and never forms as much of the cerebellar hemisphere.

In the highest primates and man the caudal expansion of the pons reaches its culmination in the complete burial of the corpus trapezoideum and of the points of attachment of the sixth, seventh, and eighth nerves. fig. 6, Plate 26. Held (1893) pointed out that in the chimpanzee and man the eighth nerves travel a considerable distance dorsal to the pons before they reach the corpus trapezoideum, and Vernon (1931) has shown that a considerable portion of the seventh nerve runs horizontally, dorsal to the caudal part of the pons, before it finally emerges from the brain-stem. The trigeminal nerve now enters the pons nearer its anterior than its posterior border.

The cerebellum has enlarged with the pons. The culmen of the lobus anterius has become further subdivided and extends laterally into the hemisphere as the anterior crescentic lobule; the declive-tuber complex has also enlarged and its lateral expansions form the bulk of the hemisphere as the posterior crescentic lobule, the superior and inferior semilunar lobules, the biventral lobule, and the tonsil. The pyramid is entirely divorced from the remains of the paraflocculi and the tonsils appear to be appendages of the uvula.

It is customary to emphasize the growth of the declive-tuber complex (lobus medius of Ingvar (1918)) of the cerebellum in response to the increase in cortical connections. But although that is undoubtedly correct, it is clear from this brief survey that the culmen of the lobus anterius has also enlarged to a considerable extent, and that this enlargement is similarly due to the increase in cortical connections. If the condition in *Echidna* is contrasted with that in the marsupial it will be seen that they are complementary: the retrotrigeminal pons of *Echidna* added to the pretrigeminal pons of the marsupial forms a completely developed pons such as occurs in the highest primates. In the cerebellum the large lobus anterius in *Echidna* added to the large lobus posterius in the marsupial approximates to the well-balanced cerebellum of the primates. It has been shown that the purely retrotrigeminal pons is associated with a hypertrophied lobus anterius; a purely pretrigeminal pons with a large lobus posterius. It has further been shown that the hypertrophy of the lobus anterius is due to the size of the culmen; that of the lobus posterius to the size of the declive-tuber complex. It thus seems certain that the retrotrigeminal part of the pons is projected chiefly on the culmen of the lobus anterius; the pretrigeminal part on the declive-tuber complex. Pathological evidence can be adduced in partial support of this statement. Biemond

(1931) found that in a case of localized infarction in the caudal part of the pons in man the resultant degeneration in the cerebellum was confined to the lobus anterius, and especially to the culmen.

It is clear that the main evolution of the pons has been from before backwards. In the marsupials and primitive true mammals the pons first appeared in front of the entering trigeminal nerves and thence extended caudally behind the fifth nerve. The Monotremes alone have pursued a peculiar course of evolution which has resulted in the development of a retrotrigeminal pons only, thus anticipating the condition in the brain of the highest primates. The reasons for this will be considered in the subsequent sections.

The Projection of the Cerebral Hemispheres on the Pons

In another paper (1934) I have shown that there is no fronto-pontine pathway in *Echidna.* The small pyramids lie at the medial border of the cerebral peduncles in close association with the substantia nigra, and the remainder of the peduncle comprises fibres which correspond to the temporo-pontine fibres of higher mammals, fig. 7, Plate 27. The absence of fronto-pontine fibres is an expression of the failure of development of any frontal cortex in the Monotremes. Martin (1898) showed that the electrically excitable motor cortex in *Ornithorhynchus* occupies the anterior two-thirds of the lateral surface of the cerebral hemisphere, and that there is no inexcitable cortex at or near the frontal pole; that is, there is no frontal cortex. In his investigation of the cortical lamination in *Echidna* Schuster (1910) found that his area 3 extended from the frontal pole over two-thirds of the lateral surface of the hemisphere to the pseudo-sylvian fissure. There is no differentiation of any frontal cortex and area 3 contains large cells of the motor type. Kappers (1933) regards area 3 in *Echidna* as the equivalent of the motor cortex in *Ornithorhynchus* and I have shown that it is from this diffuse sensory-motor area that the pyramidal tracts arise. Thus in relation to the absence of a frontal cortex there are no fronto-pontine connections, and there can be very little doubt that this lack is further reflected in the abortion of the pretrigeminal part of the pons in the Monotremes. On the other hand, there is a large temporo-pontine contribution which arises from area 4 of Schuster, and this is catered for by the retrotrigeminal pontine development.

In the marsupials and many primitive true mammals there is no retro-trigeminal pontine development, but the pons is large, fig. 2, Plate 22. Gray (1924) has shown that in the opossum *Didelphyis virginiana* there is a definite frontal cortex lying anterior to, and of histological structure different from, the excitable motor cortex. Further, Gray and Turner (1924) have demonstrated that the excitable motor cortex is restricted to the region around the fissura orbitalis—and especially to the posterior lip of this fissure—and that the histologically determined frontal cortex is inexcitable. These findings have been confirmed by Rogers (1924). Thus the marsupials possess what the Monotremes lack—a frontal cortex, and such a structure has been demonstrated in all other mammals above the Monotremes so far examined. With a frontal cortex appears a fronto-pontine pathway and it is known that this exists in all mammals used for experimental purposes for they develop decerebrate rigidity when it is interrupted. Tsai (1925) found that in the opossum the substantia nigra lies at the lateral border of the cerebral peduncle—the reverse of the condition in *Echidna*. Turner (1924) excised the motor cortex in the opossum and traced the degenerated pyramidal fibres. Although he does not describe their situation in the cerebral peduncle it is clear from his diagrams that they lie at the lateral border close to the situation of the substantia nigra as described by Tsai. Thus the position of the substantia nigra provides a rough guide to the situation of the pyramids in the peduncles. Since the pyramidal fibres in the opossum lie at the lateral border of the peduncle there can be few or no temporo-pontine fibres, for these occupy the lateral part of the cerebral peduncle when they are developed.

Unfortunately I have been unable to obtain a complete series of sections of any marsupial brain. I have examined some through the cerebral peduncle of *Dasyurus viverrinus* stained both for cells and fibres. In these, fig. 7, Plate 27, the substantia nigra can be seen at the lateral border of the peduncle. This finding confirms Tsai's observations and makes it very likely that the pyramidal fibres are situated at the lateral border of the peduncle also. Two complete series of sections through the brain of a mouse were examined. These were stained by the methods of Cajal and Ranson. In these it was possible to follow the pyramidal tracts in a reverse direction from the medulla oblongata through the pons to the peduncle with some difficulty. In these sections the interesting observation was made that, although the bulk of the non-pyramidal fibres in the peduncle are of fronto-pontine nature, a few temporo-pontine fibres can be found at the lateral border of the peduncle and these end in the most anterior part of the pons. Apart from these the pyramidal fibres occupy the most lateral part of the peduncle in close association with the greatest development of the substantia nigra, fig. 7, Plate 27, and the medial three-quarters or more of the peduncular fibres are of frontal origin.

From this comparison it appears the absence of frontal cortex and fronto-pontine fibres in the Monotremes is reflected in the aborted condition of the pretrigeminal pons, and that the temporo-pontine fibres are related to the retrotrigeminal pons and are thence relayed to the culmen of the anterior lobe of the cerebellum. In the marsupials and primitive true mammals the poor development or even complete absence of temporo-pontine fibres is expressed in the absence of any retrotrigeminal pons, the fronto-pontine fibres ending in the pretrigeminal pons and being relayed to the declive-tuber complex of the posterior lobe of the cerebellum. In view of the fact that some temporo-pontine fibres can be found in the mouse ending in the front of the pretrigeminal pons it appears that this relation is not quite absolute, and that there may be a few temporo-pontine fibres in the marsupial also.

There is some experimental evidence in favour of the view that there are few temporo-pontine fibres in lower mammals. In the cat there is a small but definite retrotrigeminal pontine development. Therefore, *a priori*, one would expect to find definite but small temporo-pontine connections. Weed (1914) and Warner and Olmsted (1923) have demonstrated experimentally that the fronto-pontine pathway is well developed in the cat. Bernis and Spiegel (1925) excised that cortical area in the cat which corresponds to the temporal region in the primates and obtained results which indicated that temporo-pontine connections exist. Warner and Olmsted stimulated electrically the cut surface of the cerebral peduncle in the cat. When they excited the medial border of the peduncle they stimulated the fronto-pontine fibres and obtained inhibition of the decerebrate rigidity, but no muscular movements. When they stimulated the lateral border of the peduncle, however, they obtained muscular movements from excitation of the pyramidal fibres. Thus in the cat the fronto-pontine fibres are sufficiently well developed to screen the pyramidal fibres in the peduncle from electrical stimulation, but the temporo-pontine fibres at the lateral border of the peduncle are so few that stimulation there excites the pyramids. Even in the cat with a definite retrotrigeminal pontine development the temporo-pontine connections are still poorly developed.

One of the most characteristic features of cerebral evolution in the higher apes and man is the progressive expansion of the cortex lying between the temporal, occipital, and post-Rolandic areas. This is associated with a steady increase in temporo-pontine connections which are laid down in the peduncle lateral to the pyramidal tracts. Thus the temporo-pontine fibres eventually come to equal the fronto-pontine fibres in bulk and the pyramids are pushed to the middle third of the crus cerebri. This is the condition which is found in man where the pyramids lie in the middle third of the crus cerebri in close relation to the greatest development of the substantia nigra, fig. 7, Plate 27, which thus appears to have migrated medially. With this increase in temporopontine connections the retrotrigeminal pons has progressively extended caudally until finally it buries the corpus trapezoideum completely. At the same time the culmen of the lobus anterius of the cerebellum has steadily enlarged.

As a result of the correlation of these two sections it seems clear that the frontal cortex is chiefly related to the pretrigeminal part of the pons whence its impulses are relayed to the declive-tuber complex of the lobus posterius of the cerebellum, while the temporal impulses are projected chiefly on the retrotrigeminal pons whence they reach the culmen of the lobus anterius of the cerebellum. It has been shown that some temporopontine fibres end in the pretrigeminal pons and, in view of the fact that the frontal cortex continues to expand during the laying down of the retrotrigeminal pons, it appears likely that some of the new frontal connections end in the retrotrigeminal pons. Thus there is some overlap of representation; this is expressed diagrammatically in fig. 8, Plate 28. The crossing of the fibres from the frontal and caudal parts of the pons to reach the caudal and frontal parts of the cerebellum respectively must take place in the brachium pontis.

The Functions of the Cortico-pontine Connections

This paper would be incomplete without an attempt to estimate the significance of the cortico-pontine connections. Our knowledge of the frontopontine tract is more complete both anatomically and physiologically than that of the temporo-pontine connection. Weed (1914) first demonstrated that injury to the fronto-pontine connection, whether in the frontal cortex, internal capsule or cerebral peduncle, induced the appearance of decerebrate rigidity which could, however, be inhibited by stimulation of the injured surface, or of the cerebellum or red nucleus. He regarded this rigidity as being homolateral as far as the fronto-pontine fibres were concerned. He also showed that section of the middle or superior cerebellar peduncle in an otherwise intact animal provoked the appearance of a similar rigidity on the same side of the body or, if the fronto-pontine connection were already cut, prevented the possibility of inhibition of rigidity by stimulation of the frontopontine fibres.

Warner and Olmsted (1923) repeated Weed's experiments and confirmed most of his conclusions, but they found that the rigidity resultant upon destruction of the fronto-pontine connection was mainly contralateral while section of the middle cerebellar peduncle exercised a homolateral effect. They concluded that the fronto-pontine fibres, after ending in the pons, gave rise to secondary fibres which passed in the brachium pontis to the opposite cerebellar hemisphere. Thence the impulses were transmitted via the brachium conjunctivum to the opposite red nucleus. From the red nucleus they cross again in the decussation of the rubro-spinal tracts before passing to the spinal cord. Therefore stimulation of the cerebellum produces homolateral inhibition of rigidity. The fronto-pontine fibres act chiefly upon the contralateral muscles, but they have, in addition, a slight homolateral effect. These results have been confirmed by Bernis and Spiegel (1925).

Kinnier Wilson (1920) has collected a large amount of clinical material bearing upon the appearance of decerebrate rigidity in man. He has shown that maldevelopment of, or injury to, the frontal cortex, or lesions in the internal capsule, mid-brain, pons or cerebellum, can produce a rigidity comparable to the decerebrate rigidity experimentally produced in animals. This rigidity may be persistent, or may take the form of tonic fits which may be complete manifestations, or may represent isolated " fragments " of decerebrate rigidity. The situation of the lesions corresponds closely with that of the fronto-pontine pathway as demonstrated experimentally by Warner and Olmsted and by Weed, and there can be no doubt that the same mechanism is affected.

Very little is known of the fibres which reach the pons from the temporal, parietal, and occipital regions of the cerebral hemispheres. Bernis and Spiegel excised the corresponding region in cats and found that there was a slight increase in extensor tone chiefly in the opposite fore-limb. They consider that this region regulates tonic labyrinthine reflexes. In view of the poor development of this area in cats and of the small size of the temporo-pontine connections, it is not surprising that the rigidity resultant upon damage in this part of the cortex was slight and confined to the fore-limbs. Kinnier Wilson showed that temporal lobe abscess produced an effect akin to decerebrate rigidity.

From the experiments of Weed, Warner, and Olmsted and Bernis and Spiegel, and the clinical findings of Kinnier Wilson, it is clear that injury to the fronto-pontine pathway alone is sufficient to produce a complete manifestation of decerebrate rigidity without the intervention of the temporo-pontine system. In rabbits in whom there is no retrotrigeminal pontine development and presumably, therefore, few temporo-pontine connections, rigidity is readily produced by section of the fronto-pontine pathway. It appears to be extremely unlikely that the temporo-pontine pathway is primarily, or even at all, concerned in the appearance of decerebrate rigidity, and another explanation for its presence must be sought.

According to Elliot Smith the function of the fronto-pontine connection is to release the muscles from the rigidity of the "standing posture" and thus render them available for any movement which the will decides to impose through the pyramidal system. This author (1927) considers that the temporo-pontine mechanism is involved in the intricate muscular co-ordination necessary for the execution of complicated movements required for play and skilled work, and for the correlation of hand and eye. Since the temporo-pontine connections are fully developed only in man in whom skilled muscular co-ordination reaches its highest expression this is undoubtedly correct, but it appears probable that the temporo-pontine system is capable of exercising yet other important functions.

The temporo-pontine fibres drain an area which in lower mammals is closely circumscribed by the primary sensory receptive areas for touch, vision, hearing, and, probably, equilibration. This region shows the greatest expansion in the primates and becomes complexly differentiated. At the same time the temporo-pontine connections increase progressively in number. In this expanding region develop the visuo-auditory and visuo-tactile bands of Elliot Smith who has shown how important has been the co-ordination of hand and eye in the evolution of the macula lutea and stereoscopic vision, and the resultant dominance of the primates. Certainly, then, the temporo-pontine connections are favourably situated to effect the necessary co-ordination of eye and hand muscles, and their steady increase in the primates enhances the possibility of such co-ordination. They are also in a position to correlate such movements in response to tactile impressions and thus reinforce the information gained from visual examination. In man rhythmic movements in time to music have reached a high stage of expression; again the temporo-pontine fibres are able to effect the necessary co-ordination between hearing and muscular movements. In man sports demanding the most accurate muscular control of balance, for example, skating, depend upon reaction to vestibular, proprioceptive, tactile, and visual impressions, and these can only be correlated satisfactorily in the region which lies between the primary receptor areas for these senses; that is, the region from which the temporo-pontine fibres arise. Thus in man and, to a lesser extent, in the higher primates, the temporo-pontine connections are developed in equal relation to all the important senses and provide the opportunity for well-balanced muscular response to the resultant.

In the Monotremes, however, the temporo-pontine pathway is extraordinarily developed ; and this in animals in whom sight is almost atrophied, and hearing little better than in birds. Also, it has already been noted that there is a precocious appearance of a retrotrigeminal pons in several very primitive mammals. *Notoryctes typhlops* possesses no eyes at all and its cochlea has advanced only to a slight degree upon that in the Monotremes. The hedgehog, mole, agouti, and the edentates cannot be regarded as exponents of skilled muscular co-ordination or the close correlation of touch and vision since the mole, at least, is blind. Yet all these animals betray evidence of requiring temporo-pontine control. Nor can hearing be invoked for in most marsupials the auditory apparatus is fully as well developed as it is in the highest mammals, but there are few, if any, temporo-pontine connections.

The Monotremes, *Notoryctes typhlops,* the hedgehog, mole, agouti, and the edentates all have one feature in common : they all live either in burrows or in close contact with the ground. Under such circumstances there is one sense which, together with smell, is of more importance than sight or hearing : the sense of vibratory perception. It is probable that vibratory impulses transmitted through the ground may be perceived both by tactile and by vestibular sense. In the case of the Monotremes tactile sensibility of the body as well as of the snout is extraordinarily well developed, in the case of the other animals it is possible that vibratory perception is mainly through the vestibule. In either case it becomes represented cortically in close relation to the origin of the temporo-pontine fibres which thus provide a means for immediate muscular reaction in response to vibrations. In the Monotremes vibratory perception apparently plays such a large part in their existence that they have evolved their powers of muscular response in relation particularly to this sense. Wood Jones (1923) has emphasized the extraordinary development of the tactile sense of vibrations in *Echidna* and it seems probable that the Monotremes are unique in their development of this sense to the exclusion of all others except smell.

Thus it appears that the fronto-pontine and temporo-pontine tracts are complementary in their functions. The function of the fronto-pontine pathway appears to be the release of the muscles from the economical rigidity of the standing posture immediately the will decides to alter that posture. But the function of the temporo-pontine pathway is infinitely more subtle. It undertakes the task of weaving all sensory impulses—tactile, proprioceptive, equilibratory, vibratory, auditory, and visual—into a homogeneous fabric and translating the resultant in muscular response which is accurately co-ordinated and acutely adapted to the requirements of the situation as a whole, no matter how complex it may be. It is only with the attainment of a balanced maturity of this mechanism in equal relation to all avenues of information concerning the situation in hand that the fullest possibilities of muscular co-ordination can be realized. To it man owes the possibility of his highest powers as expressed in his work, in sport, and in art.

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Summary

1—In the Monotremes there is no frontal cortex. As a result there is complete absence of the fronto-pontine tract, abortion of the pretrigeminal part of the pons and failure of expansion of the declive-tuber complex in the lobus posterius of the cerebellum. But the temporo-pontine tract is well developed and has provoked the development of a retrotrigeminal pons and hypertrophy in the culmen of the lobus anterius of the cerebellum.

2—In practically all marsupials, and in many primitive eutheria, there are few or no temporo-pontine fibres in the cerebral peduncle, no retrotrigeminal pontine development and a rudimentary culmen in the lobus anterius of the cerebellum. But there is a well-developed frontal cortex which gives rise to a fronto-pontine tract, a pretrigeminal pontine development and a large declive-tuber complex in the lobus posterius of the cerebellum.

3—In higher sub-primates there is a small but definite increase in the number of temporo-pontine connections and a resultant appearance of a small retrotrigeminal pontine development. This is reflected in the enlargement of the culmen in the lobus anterius of the cerebellum.

4—In the primates there is a steady increase in the number of temporopontine connections accompanied by a progressive extension of the retrotrigeminal pons and a continuous expansion of the culmen in the lobus anterius of the cerebellum.

5—It is considered that the fronto-pontine fibres end chiefly in the pretrigeminal part of the pons whence their impulses are relayed to the declive-tuber complex of the lobus posterius of the cerebellum. The temporo-pontine fibres end chiefly in the retrotrigeminal part of the pons and are relayed to the culmen of the lobus anterius of the cerebellum. A few temporo-pontine fibres end in the pretrigeminal part of the pons--presumably to be relayed to the declive-tuber complex, while some fronto-pontine fibres probably reach the retrotrigeminal part of the pons to be relayed to the culmen.

6—The fronto-pontine pathway appears to be related to the release of muscles from the rigidity of the standing posture when they are required for action. The temporo-pontine connections appear to have evolved in response to the need for co-ordinated muscular reaction to the information received by the tactile, proprioceptive, equilibratory, vibratory, auditory, and visual senses. In some mammals one or more of these avenues of information may be developed to the neglect of the others.

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EXPLANATION OF PLATES

Key to Lettering

ce. ped., cerebral peduncle. *corp. trap.*, corpus trapezoideum *cu.*, culmen. *d.t.c.*, declive-tuber complex. *fiss. p.*, fissura prima. *fiss. præpy.*, fissura præpyramidalis. *fiss. s.*, fissura secunda. *fl.*, flocculus. *fr.p.f.*, fronto pontine fibres. *fr. cor.*, frontal cortex. *fr. p. tr.*, fronto-pontine tract. *la. lem.*, lateral lemniscus. *li.*, lingula. *lo. cen.*, lobulus centralis. *m. cor.*, motor cortex. iii N., third nerve. *V N.*, fifth nerve. *VI N.*, sixth nerve. *VII N.*, seventh nerve. *VIII N.*, eighth nerve. *nd.*, nodule. *occ. cor.*, occipital cortex. *p.*, pons. *par. cor.*, parietal cortex. *pa. fl.*, paraflocculus. *pre. p.*, pretrigeminal pons. *py.*, pyramid. *py. f.*, pyramidal fibres. *py. tr.*, pyramidal tract. *r. p.*, rostrum pontis. *red nu.*, red nucleus. *ret. p.*, retrogeminal pons. *sub. nig.*, substantia nigra. *ton.*, tonsil. *tem. cor.*, temporal cortex. *t. pon. f.*, temporo-pontine fibres. *t. p. tr.*, temporo-pontine tract. *u.*, uvula. *v. teg. nu.*, ventral tegmental nucleus.

PLATE 21

FIG. 1—Ventral, median sagittal, and dorsal views of the brain-stem and cerebellum of *Echidna*. × 3. Note the purely retrotrigeminal development of the pons and the enormous size of the culmen of the lobus anterius of the cerebellum. The lobus posterius is stippled. In the dorsal view of this and other cerebella the declive-tuber complex is enclosed within the heavy line.

PLATE 22

FIG. 2—Ventral, median sagittal, and dorsal views of the brain-stem and cerebellum of a marsupial, *Macropus thetidis*. × 3. Note the purely pretrigeminal development of the pons and the hypertrophy of the declive-tuber complex of the lobus posterius of the cerebellum, especially from the dorsal aspect.

Abbie.

Proc. Roy. Soc., B. vol. 115, Plate 21.

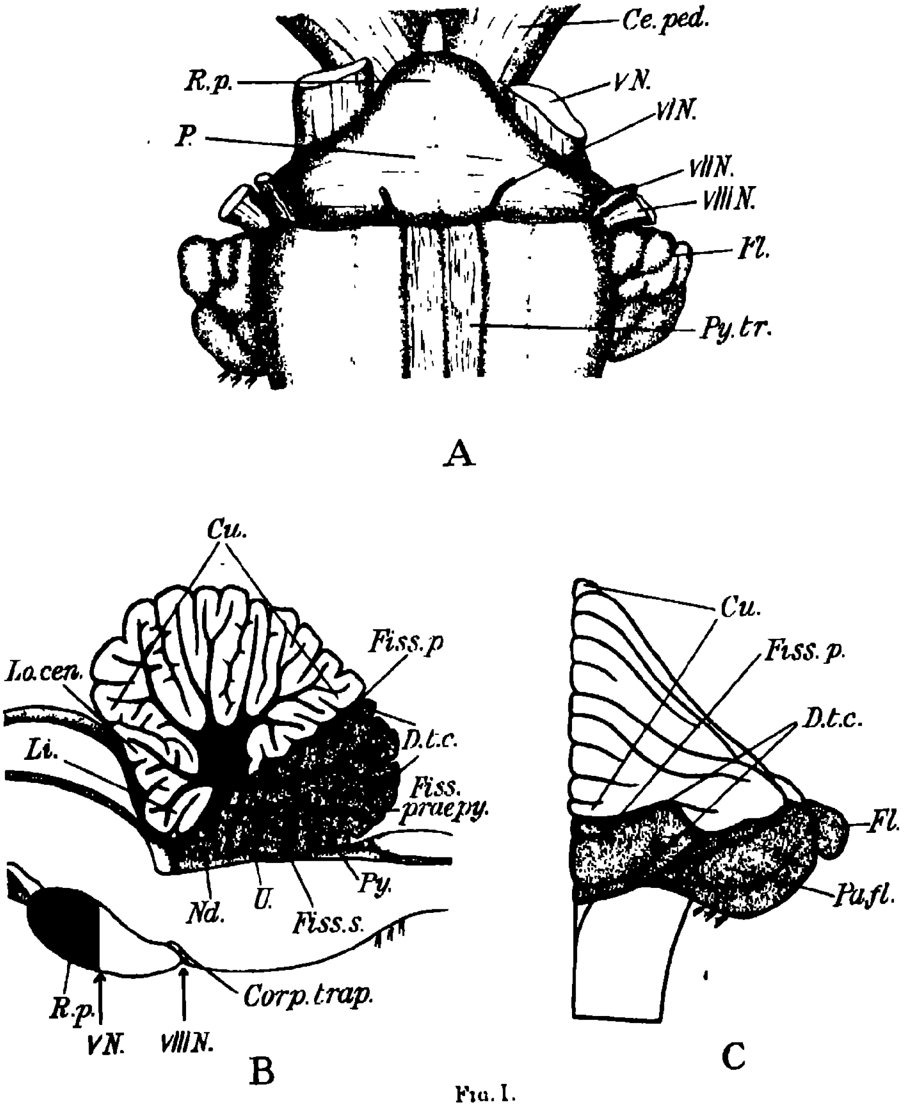


Fig. I.

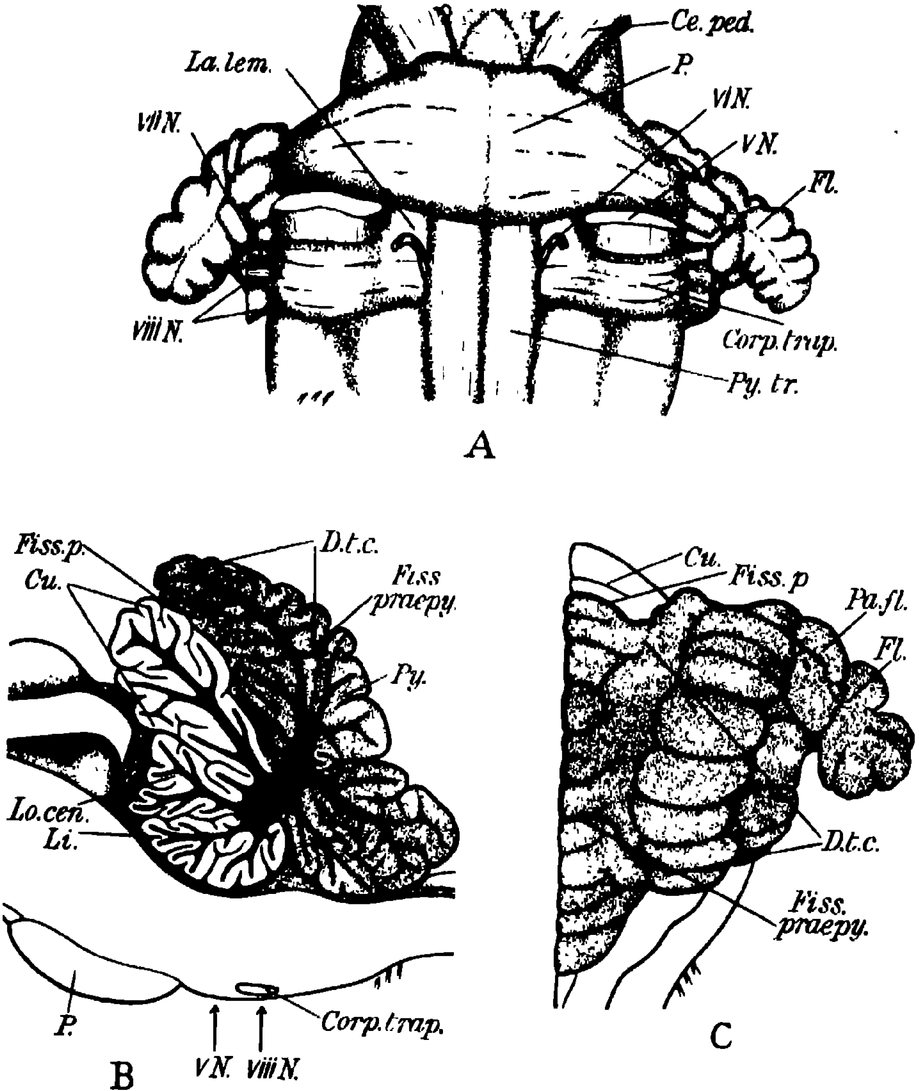

Ce. ped.
La. lem.
P.
VI N.
VII N.
V N.
Fl.
VIII N.
Corp. trap.
Py. tr.
A
Fiss.p.
D.t.c.
Cu.
Fiss praepy.
Py.
Lo. cen.
Li.
P.
V N.
VIII N.
Corp. trap.
B
Cu.
Fiss. p
Pa. fl.
Fl.
D.t.c.
Fiss. praepy.
C

Fig 2.

Abbie.

Proc Roy. Soc., B. vol. 115, *Plate* 23.

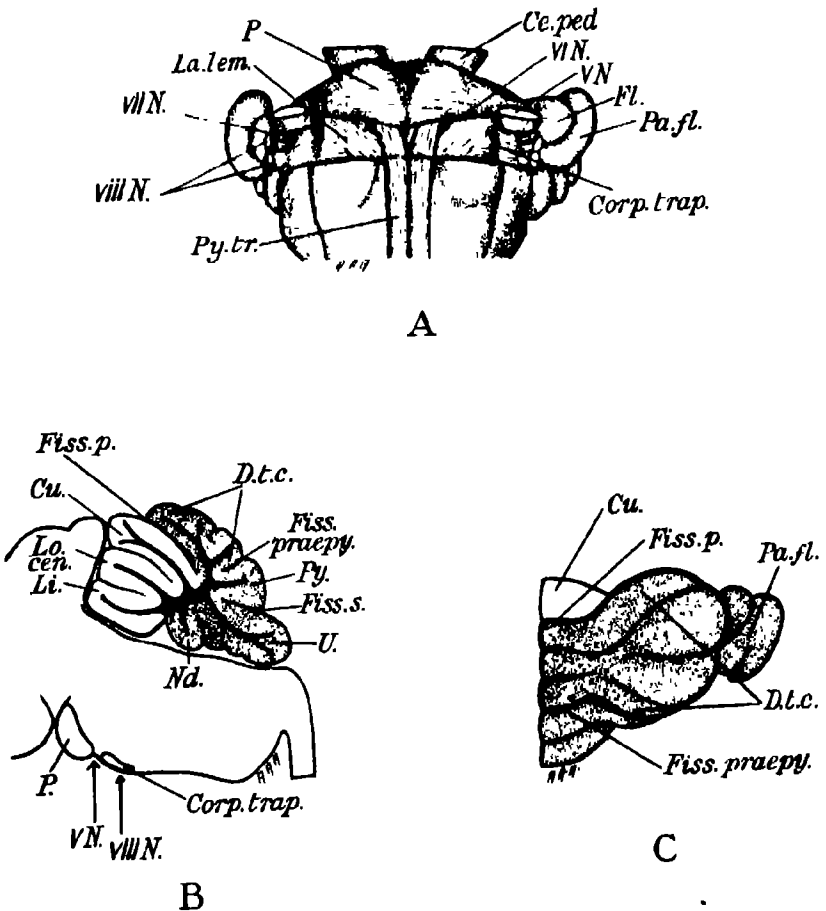
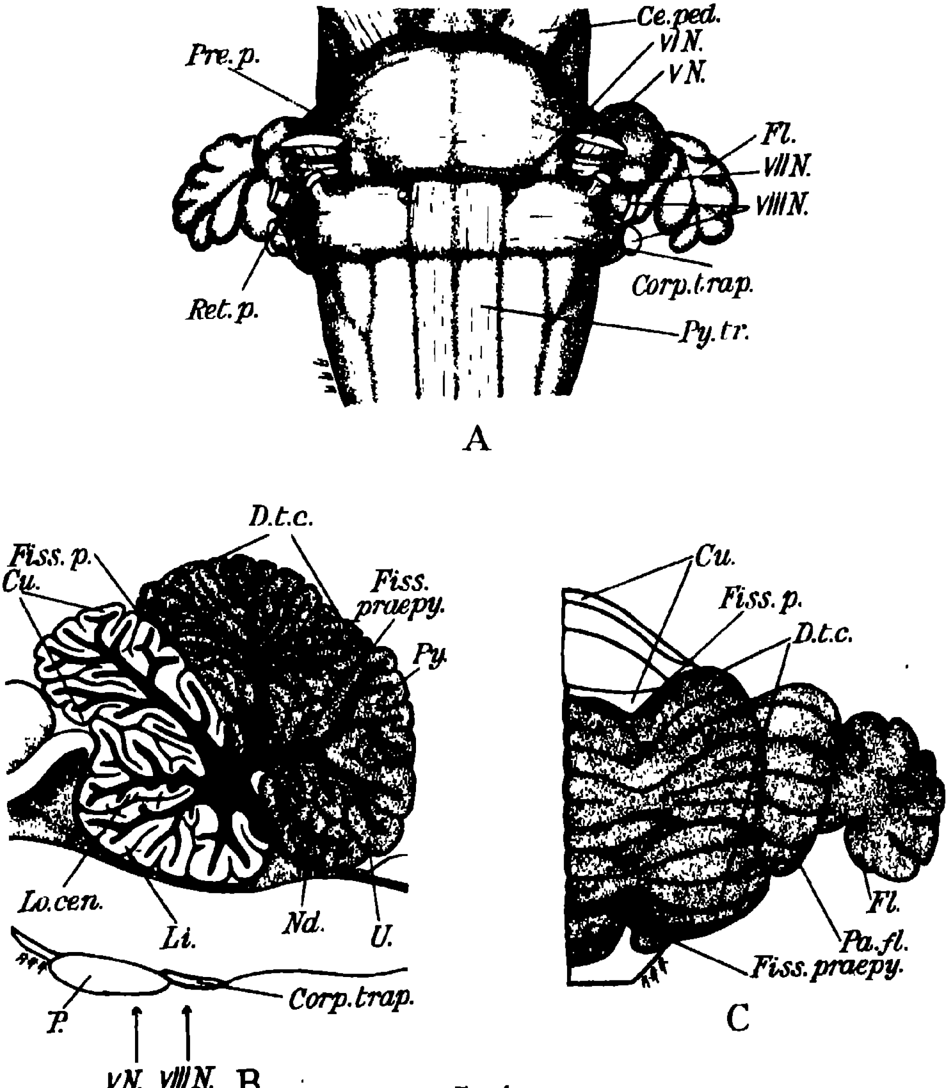


Fig 3.


Ce.ped.
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VI N.
V N.
Fl.
VII N.
VIII N.
Corp.trap.
Ret.p.
Py.tr.
A
D.t.c.
Fiss. p.
Cu.
Fiss. praepy.
Py.
Lo.cen.
Li.
Nd.
U.
P.
Corp.trap.
V N.
VIII N.
B
Cu.
Fiss. p.
D.t.c.
Fl.
Pa.fl.
Fiss. praepy.
C

Fig. 4.

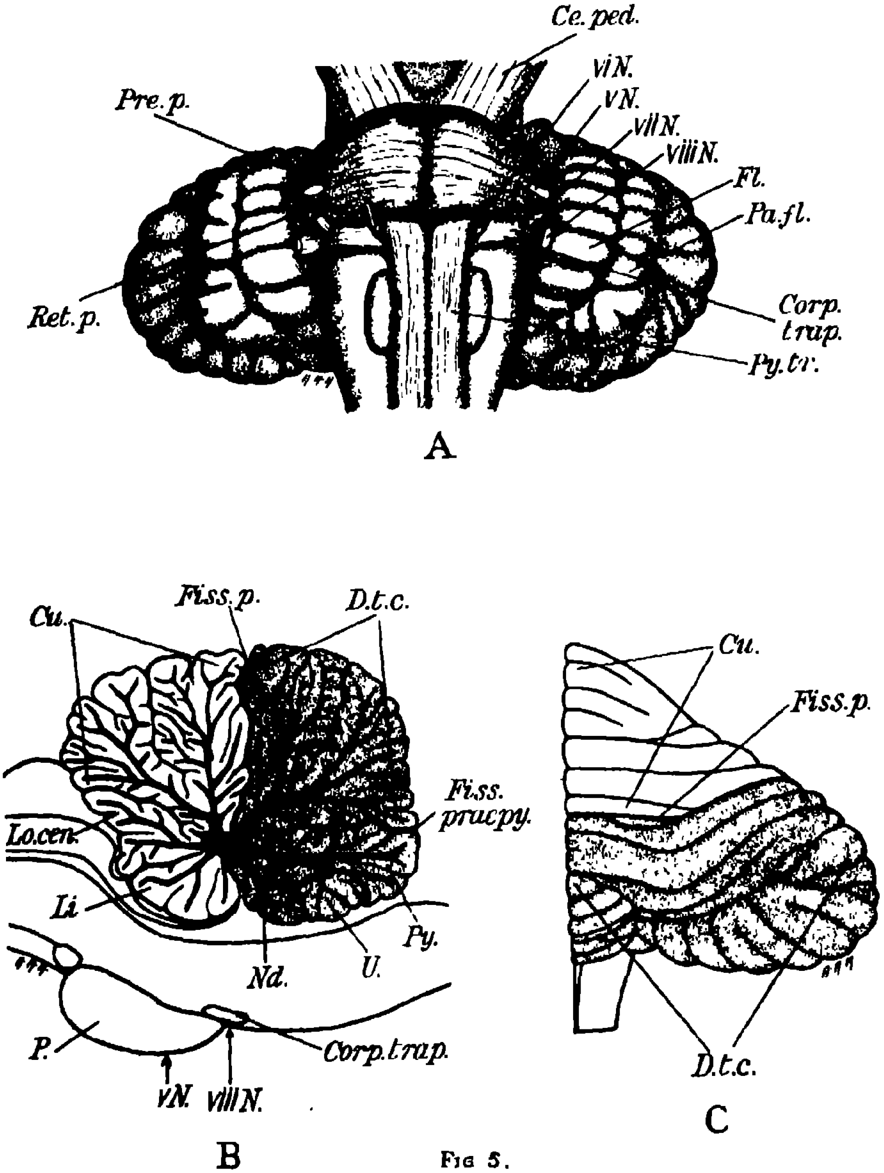
Ce. ped.
vi N.
v N.
vii N.
viii N.
Fl.
Pa. fl.
Pre. p.
Ret. p.
Corp. trap.
Py. tr.
A
Cu.
Fiss. p.
D.t.c.
Fiss. prucpy.
Lo. cen.
Li
Nd.
U.
Py.
P.
v N.
viii N.
Corp. trap.
B
Cu.
Fiss. p.
D.t.c.
C

Fig 5.

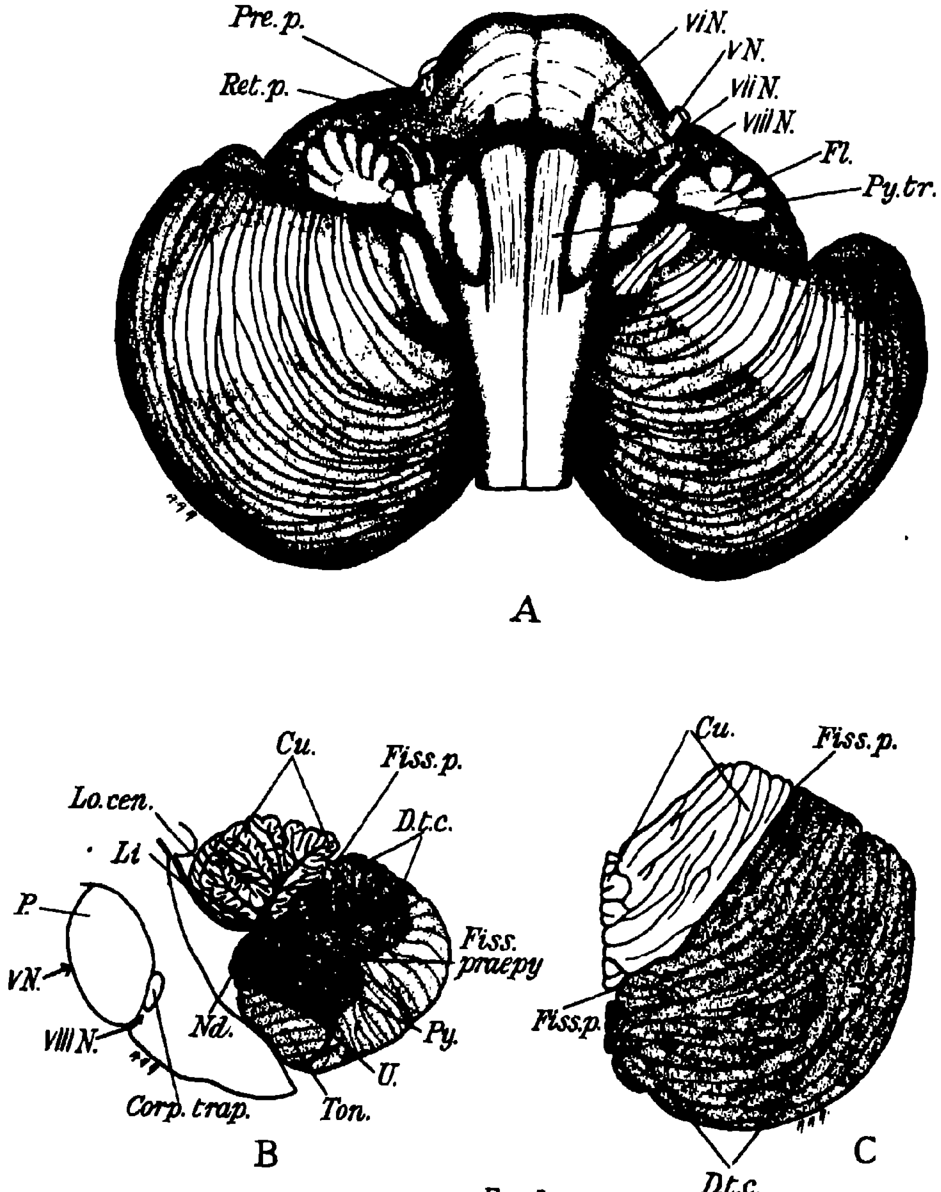
Pre.p.
Ret.p.
VIN.
VN.
VIIN.
VIIIN.
Fl.
Py.tr.
A
Cu.
Fiss.p.
Lo.cen.
Li
D.t.c.
P.
Fiss. praepy
VN.
VIIIN.
Nd.
Py.
U.
Ton.
Corp. trap.
B
Cu.
Fiss.p.
Fiss.p.
D.t.c.
C

FIG. 6.

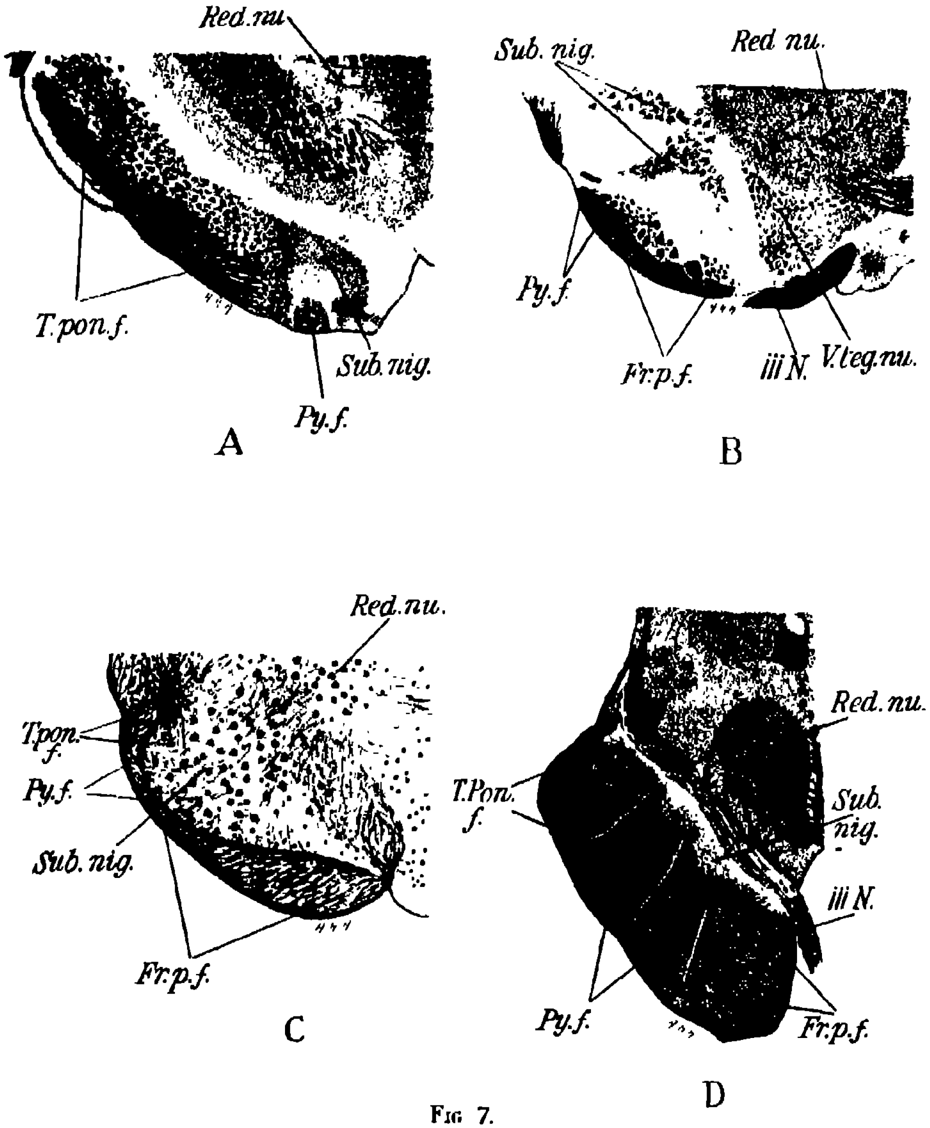
Red.nu.
T.pon.f.
Sub.nig.
Py.f.
A
Sub. nig.
Red nu.
Py.f.
Fr.p.f.
iii N.
V.teg.nu.
B
Red.nu.
T.pon.f.
Py.f.
Sub. nig.
Fr.p.f.
C
Red.nu.
T.Pon.f.
Sub.nig.
iii N.
Py.f.
Fr.p.f.
D

Fig. 7.

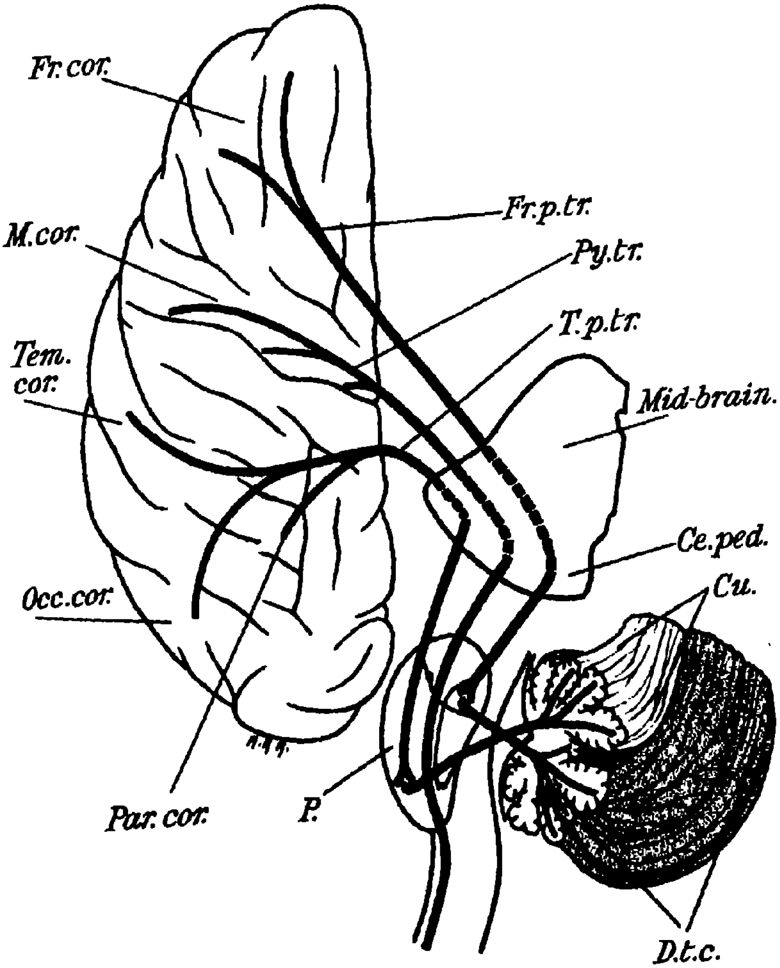
Fr. cor.
M.cor.
Tem. cor.
Occ.cor.
Par. cor.
P.
Fr.p.tr.
Py.tr.
T.p.tr.
Mid-brain.
Ce.ped.
Cu.
D.t.c.

Fig. 8.

Plate 23

Fig. 3—Ventral, median sagittal, and dorsal aspects of the brain-stem and cerebellum of the mouse. × 3. Note that although the pons is still purely pretrigeminal in development it has extended caudally in comparison with that of the marsupial. The declive-tuber complex of the lobus posterius of the cerebellum still forms the bulk of this organ.

Plate 24

Fig. 4—Ventral, median sagittal, and dorsal aspects of the brain-stem and cerebellum of another rodent, the golden agouti, *Dasyprocta aguti*. × 3. The pons has developed a small retrotrigeminal portion and is now covering the frontal border of the corpus trapezoideum. At the same time the culmen of the lobus anterius of the cerebellum has enlarged considerably, this is especially obvious from the dorsal view.

Plate 25

Fig. 5—Ventral, median sagittal, and dorsal views of the brain-stem and cerebellum of a monkey, *Cercopithecus sabaeus*. × 2. The retrotrigeminal pons has increased considerably in size and now covers a large portion of the frontal part of the corpus trapezoideum. The culmen has also enlarged to a great extent and in median section almost equals the declive-tuber complex in bulk. But from the dorsal view it can be seen that the total bulk of the culmen is still considerably inferior to the total bulk of the declive-tuber complex.

Plate 26

Fig. 6—Similar views of the human brain-stem and cerebellum, × ¾. The retrotrigeminal pons now exceeds the pretrigeminal pons in extent and covers the corpus trapezoideum completely. Its relation to the sixth, seventh, and eighth nerves is the same as in *Echidna*. The culmen of the lobus anterius nearly equals the declive-tuber complex in size in the median section, but the total bulk of the culmen, as seen from the dorsal aspect, is considerably inferior to that of the declive-tuber complex.

Plate 27

Fig. 7—(*a*) Tranverse section through the cerebral peduncle of *Echidna*. × 7. Weigert-Pal stain. Note that the pyramidal fibres and substantia nigra are confined to the medial border of the peduncle and that the rest of the peduncle comprises temporo-pontine fibres. (*b*) Section through the peduncle of *Dasyurus viverrinus*. × 14. Stain unidentified. The pyramids and substantia nigra are situated at the lateral border of the peduncle the remainder of which comprises fronto-pontine fibres. (*c*) Section through the peduncle of the mouse. × 14. Ranson's stain. A few temporo-pontine fibres have appeared at the lateral border of the peduncle and the pyramidal fibres and substantia nigra appear to have migrated somewhat medially. (*d*) Section through the human cerebral peduncle. × 3. Weigert-Pal stain. The temporo-pontine fibres now equal in bulk the fronto-pontine fibres, and the pyramids. The greatest concentration of the substantia nigra occupies the middle third of the peduncle.

PLATE 28

FIG. 8—Diagram of the origin, course, and distribution of the fronto-pontine, parieto-pontine, and temporo-pontine fibres and the pyramidal tracts. Note that while the bulk of the fronto-pontine fibres end in the pretrigeminal part of the pons to be relayed to the declive-tuber complex of the cerebellum, a few pass to the retrotrigeminal pons to reach the culmen of the lobus anterius. On the other hand, most of the temporo-pontine fibres terminate in the retrotrigeminal pons to be relayed to the culmen of the lobus anterius, but a few end in the front of the pons to reach the declive-tuber complex of the lobus posterius.

631.476 576.809.34

The Numbers of Bacterial Cells in Field Soils, as Estimated by the Ratio Method

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1. *Introduction*

It has long been recognized that the method of counting bacteria in a soil sample by means of plate counts is valueless as an estimate of the total bacterial population, although such counts have undoubted value in comparing two or more samples. The numbers obtained by this method represent only a small fraction of the total population. This is due mainly to the fact that no single plating medium will enable all the diverse groups of soil bacteria to multiply and produce colonies. Indeed, many types will not grow on any of the media commonly used for plate counts, and it is very uncertain whether those which do so are of main importance in carrying out biochemical changes in the soil itself. The number of colonies which appear is probably reduced further by the fact that, in some soils, many of the bacteria occur in clumps which would not easily be broken up in the process of making the soil suspension. These limitations of the plate method make it very important that a technique should be developed for making accurate estimates of the total bacterial content of a soil sample from direct microscope counts.

The counting of bacteria in dried and stained films has become a recognized technique for milk (Breed, 1911). A comparison between plate and direct microscope counts of milk samples was made by Breed and Stocking (1920) and more recently by Buice (1934) who found that the numbers obtained by two methods were of the same order. With milk, however, most, if not all, of the organisms counted were of types capable of growing upon the plating medium used. The application of direct counting methods to bacteria in a soil sample is greatly complicated by the presence of the soil particles. These introduce difficulties in staining the film, in obtaining an even distribution of bacteria over the film, and in estimating the mass of soil examined.

The difficulty in obtaining a differential stain for the bacteria in a soil film is due to the existence of particles of organic material which take up most of the stains commonly used for bacteria. Much of the soil organic matter consists of "humic acid" compounds which combine with the common basic dyes. Conn and Winogradsky found, however, that if an acid dye were employed, these bodies took up little or none of the stain, so that a differential stain for the micro-organisms could be obtained. Conn (1918) employed Bengal rose, but Winogradsky (1925) found that erythrosine gave a clearer stain. A modification of Winogradsky's staining method is employed in the technique here discussed.

By staining dried films of soil suspensions with these acid dyes, Conn, and especially Winogradsky, have made some very interesting qualitative observations on the appearance of bacteria in the soil itself thus opening upon a new field in soil microbiology. Koffmann (1928) developed a different and somewhat cumbersome technique in which a soil suspension, placed between a cover glass and slide, was fixed, stained, dehydrated, "cleared," and finally mounted in balsam by diffusion of the various liquids in from the edges of the cover glass. This method may have value for the qualitative study of delicate soil organisms that are destroyed in a dried film. It would seem too lengthy to form the basis of a quantitative method.

The preparation of dried and stained films of a soil suspension, however, is rapid and simple, and the use of such films for estimating the numbers of bacteria in soil naturally suggested itself. Indeed both Conn and Winogradsky have devised such methods.

Conn (1918) used a direct application of Breed's method already in use for counting bacteria in milk. One hundredth of a cubic centimetre of soil suspension of known dilution was spread over a square centimetre of microscope slide. The film was dried and stained and the bacteria in random microscope fields of known area were counted. Direct calculation from the mean count gave the numbers in the original film and suspension. An important factor limiting the accuracy of this method must be the difficulty in obtaining an accurate sample of a suspension of soil particles only 0·01 cc in volume. Any error in the quantity of soil sampled will be multiplied by a large factor in calculating the numbers per gram. Winogradsky (1925) sought to estimate the quantity of soil in the film by weighing. Here again the error involved in weighing a soil film of perhaps 0·1 mg must introduce a large source of error in the final estimate of numbers per gram.

In both methods the calculation of the number of organisms per gram of soil involves an estimate not only of the small mass of soil in the film, but also of the number of bacteria in that film. Unfortunately this latter estimate is also liable to a grave source of error. It is based on the mean count of a number of random microscope fields and its reliability consequently depends on the assumption that the distribution of organisms over the film is random. An extensive series of counts which were made by Kühlmorgen-Hille (1928) enables this point to be tested. He made bacterial counts from 19 different soils using both Conn's and Winogradsky's methods and also the plate method. The two direct methods gave very different results. The actual counts obtained from individual microscope fields by each method are given and have enabled the present authors to test the agreement between such replicate counts within each sample by calculating the indices of dispersion according to the statistic

$$\chi^2 = S\,\frac{(x-\bar{x})^2}{\bar{x}},$$

where x = any individual count, $\bar{x}$ = their mean and S stands for summation (Fisher, 1932). Where the distribution of the counts agrees with the expectation of random sampling, the value of χ^2 should approximate to the number of degrees of freedom. When using Conn's method, Kühlmorgen-Hille made counts of 40 parallel microscope fields from each of the 19 soils, thus obtaining a series of counts having 39 × 19 = 741 degrees of freedom. The values of χ^2 for the 19 sets total 1096·16, a value which is quite outside the expectations of random sampling. Winogradsky's technique involves the division of each soil sample into five fractions by sedimentation and centrifuging.* Kühlmorgen-Hille made counts from 24 random microscope fields from each of the five fractions of his 19 soil samples, thus providing 95 sets of counts, each having 23 degrees of freedom, from which to estimate the dispersion between replicate microscope fields. Of these, however, six sets gave such low counts, with many blank fields, that no valid estimation of χ^2 could be obtained from them. Table I shows the distribution of the values of χ^2 among the remaining 89 sets, together with the distribution to be expected in 89 sets of random samples. Column 2 shows for each value of χ^2 the corresponding magnitude of P, which is the percentage probability that a given value will be exceeded in a random sample. Here again there is a large excess of high values of χ^2, indeed in 24 of the sets the values are larger than could occur once in a hundred random samples. One must conclude, in default of contrary evidence, that the bacteria in a dried film of soil are distributed so unevenly that *direct* counts from random microscope fields do not provide valid samples of the whole film, and therefore do not provide reliable data from which to calculate the total bacterial content of that film. This conclusion, derived from Kühlmorgen-Hille's data, is confirmed by our results, given below.

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* This division of the sample complicates the estimate of bacterial numbers owing to uncertainty as to the relative value to be assigned to counts from each fraction.

Table I—χ^2 indices from 89 sets of 24 replicate microscope field counts using Winogradsky's method (from Kühlmorgen-Hille's data).

χ^2 ($n = 23$)	P	Number of sets observed	Number of sets expected
		2	0·89
10·12	0·99		
		2	0·89
11·29	0·98		
		2	2·67
13·09	0·95		
		2	4·45
14·85	0·90		
		4	8·9
17·19	0·80		
		2	8·9
19·02	0·70		
		13	17·8
22·34	0·50		
		8	17·8
26·12	0·30		
		3	8·9
28·43	0·20		
		7	8·9
32·01	0·10		
		10	4·45
35·17	0·05		
		6	2·67
38·97	0·02		
		4	0·89
41·64	0·01		
		24	0·89

There are thus two serious difficulties in estimating the bacterial numbers in a soil sample from the examination of stained films of that soil. The ***first*** is that of determining with sufficient accuracy the mass of soil in the film, the ***second*** that of estimating, from random microscope fields, the total number of bacteria in the film, when these are not in fact distributed through it at random.* It seemed possible that these difficulties might be circumvented by the use of a *ratio* method similar to that devised by Wright (1912) in which a counted suspension of blood corpuscles is used to determine the density of a bacterial suspension mixed therewith. The principle of this method as applied to soil is as follows. A suspension of particles of distinctive colour is made up and the number of particles per cubic centimetre is determined. A known mass of soil is shaken up in a known volume of this suspension and films of the resulting mixture are prepared, dried, and stained. Counts are made of bacteria and of added particles in random microscope fields and the ratio of one to the other is determined. Since the absolute number of particles added per gram of soil is known, the number of bacteria is simply calculated. This method avoids the necessity of estimating the mass of soil in the film, since the calculation of bacterial numbers from the ratios depends upon the mixing of soil and particle suspensions in quantities large enough to measure with ease and accuracy. Moreover, the uneven distribution of bacteria over the dried film is mainly due to surface tension disturbances. So that, if coloured particles of the same size and density as bacteria are employed, it seemed probable that bacteria and added particles would be similarly acted on by surface tension forces, and that the ratio between them over different areas of the film would remain undisturbed during drying. One should thus obtain a more even distribution of ratios than of bacteria taken by themselves.

The properties required of coloured particles greatly restricts the range of substances that can be employed. They must be --

1. Insoluble in water.
2. Unaltered by drying.
3. Unaffected by the stain used for the bacteria.
4. Of a bright colour contrasting with this stain.
5. Transparent, so that the colour may show by transmitted light.
6. Of approximately the specific gravity and size of bacteria.

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* The attempt to avoid the difficulties due to soil in the film was made by Vande Velde and Verbelen (1930). This method consists in shaking the soil with sterilized milk, plus a trace of formaldehyde, allowing it to settle and counting the bacteria in films of the supernatant fluid. The present authors know of no data enabling them to judge what relationship exists between the bacterial numbers in the supernatant milk and those in the original soil sample. A method somewhat similar in principle, in which counts are made from an alkaline soil suspension after centrifuging, has been evolved by Germanov (1932).

After trying many substances it was found that indigotin was the most satisfactory substance and that, in sufficiently small particles, it possessed all the above qualities.

2. *Technique*

The technique finally developed was as follows* :—

A—Preparation of the Indigotin Suspension—Half a gram of finely powdered indigotin is well shaken in 500 cc of sterile distilled water and the suspension is passed rapidly through coarse filter paper (*e.g.*, Whatman No. 4). The paper should be changed as soon as the pores show signs of becoming choked. The suspension is then counted on a hæmocytometer, 0·02 mm in depth. There should be about 500 million particles per cubic centimetre and their mean diameter should be about 1·5 μ. Unless the suspension is used at once, 0·2 gram of $HgCl_2$ per litre should be added to prevent bacterial growth. It is well to examine a stained smear of the suspension to ensure that it contains no stainable organisms.

Filtration does not ensure such uniformity in particle size as would be ideal. A great advantage would ensue if particles of more uniform size could be obtained. Satisfactory suspensions have been made by Dr. Hugh Nicol by chemical methods, but, hitherto, undesirable bye-products have caused frothing in the presence of soil.

B—Preparation and Staining of the Films—The soil to be examined is passed through a 3 mm sieve and 5 grams are shaken with 25 cc of the counted indigotin suspension for 3 minutes.† The mixture is then diluted with 25 cc of sterile 0·01% agar and shaken for a further 3 minutes. The agar assists the subsequent adhesion of the film to the slide surface. About 5 cc of the mixture is quickly poured into a specimen tube for greater ease of manipulation. Five drops of about 2 mm diameter are placed on the surface of a carefully cleaned microscope slide by means of a mapping pen, the tube being shaken between the making of each drop. The drops are then left to dry. Such small drops dry rapidly and with a minimum of disturbance due to surface tension. Four or five replicate slides should be prepared from each soil sample. The slides are stained for 10 minutes in 5% erythrosine dissolved in 5% phenol, and restained for 10 minutes in saturated aqueous erythrosine washing in distilled water after each stain. The slides are then dried. The second staining intensifies the colouring of the bacteria. Other acid dyes can be used. Bengal rose, phloxine, cyanosine, and acid fuchsine have been tested but have not given better results than erythrosine.*

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* A note describing the method was published in 'Nature,' vol. 122, p. 400 (1928).

† Soils very poor in bacterial cells should be less diluted.

C—*Method of Counting*—The micro-organisms and indigo particles in a number of random microscope fields are counted under an oil immersion. It is best to insert in the eye piece a glass disc ruled with a square having 2 mm sides and to take only the area covered by this square as a field. Accuracy of counting is notably increased and eye fatigue reduced by the use of a binocular microscope. The number of fields to be counted depends upon the degree of accuracy required and upon the numbers of bacteria and indigo particles found per field (see below, p. 531). Where five drops on each of four slides are examined, it is usually sufficient to count 4 fields per drop so that the estimate of numbers is based on 80 fields. An important advantage of such microscope counts is that the preparations are permanent, so that the accuracy of a given estimate can be increased at any time by counting a greater number of fields. The number of organisms, o, per gram of soil is calculated according to the formula

$$o = 5y \cdot \frac{B}{I},$$

where y = the number of indigo particles per cubic centimetre of the suspension 5 cc of which were added per gram of soil, and B and I the total numbers respectively of organisms and indigo counted in the films. A typical series of counts is illustrated in Table II.

3—*Tests of the Method*

(*a*) *Agreement between Replicate Microscope Fields and Drops*—A serious source of error in other proposed microscope methods of counting soil organisms has been the uneven distribution of these organisms over the film. It was hoped that, in the present method, *ratios* of organisms to added particles might be more uniform over the film than was the distribution of the organisms taken by themselves. This can be tested by calculating the χ^2 indices of dispersion between replicate microscope fields. (For method of computating the ratio χ^2, see Appendix.) These values have been calculated for nine series of counts from soils of three Barnfield plots. The results are shown in Table III where column A gives the number of degrees of freedom, n, for each count. Columns B and C show the χ^2 indices and the corresponding

---

* There is some confusion as to the terminology of the fluorescein group of dyes. The stain used is "*erythrosine B*," (tetra-iodo-fluorescein).

Table II—Count of Micro-organisms from Sample of Soil from Barnfield Plot 1-0

	Slide 1								Slide 2							
Drop No.:	1		2		3		4		5		6		7		8	
	I	B	I	B	I	B	I	B	I	B	I	B	I	B	I	B
Microscope fields 1	14	28	7	23	8	20	14	17	9	21	8	14	7	11	5	9
2	18	33	12	23	8	12	5	11	19	36	8	11	10	18	9	24
3	8	14	13	27	8	18	7	12	13	15	11	16	13	17	12	10
4	11	21	8	15	13	20	16	14	13	18	10	13	7	23	9	11
Indigo counted per drop..	51		40		37		42		54		37		37		35	
Organisms counted per drop......		96		88		70		54		90		54		69		54

	Slide 3								Slide 4								
Drop No.:	9		10		11		12		13		14		15		16		
	I	B	I	B	I	B	I	B	I	B	I	B	I	B	I	B	
Microscope fields 1	10	27	5	13	8	9	18	20	10	18	5	13	4	11	10	16	χ^2 of drop ratios = 9·27.
2	5	9	12	18	3	10	9	10	4	12	9	17	11	13	13	21	χ^2 of drops, organisms alone = 55·91.
3	10	13	8	9	6	18	12	18	10	15	5	11	8	8	3	9	
4	11	17	10	7	7	14	7	15	11	24	12	9	7	15	13	18	$n = 15$.
Indigo counted per drop..	36		35		24		46		35		31		30		39		Total indigo counted, 609.
Organisms counted per drop......		66		47		51		63		69		50		47		64	Total bacteria counted, 1032.

Indigo particles per cc of added suspension—380·8 millions
Micro-organisms per gram of soil—3226·5 millions.

values of P for the counts of micro-organisms taken by themselves. Here, as with Kühlmoren-Hille's material, the micro-organisms are unevenly distributed over the films, the values of χ^2 being excessive in six of the nine sets, and in two of these being extremely high. Columns D and E show the χ^2 indices with their values of P for the *ratios* derived from the same replicate microscope fields. In only one set does the value of χ^2 exceed that expected in a random sample. Thus, although the micro-organisms were unevenly distributed over the films, the *ratios* of micro-organisms to indigo particles from the same microscope fields did not in general show excessive variability. This desirable result must be attributed to the surface tension forces acting similarly upon the micro-organisms and upon the indigo.

Table III—χ^2 indices of dispersion for ratios of organisms to indigo particles and for organisms alone in replicate microscope fields

	A	B	C	D	E
Soil	n	χ^2 of organisms alone	P	χ^2 of ratios	P
Barnfield plot 8–O—					
Sample 1	63	55·82	0·74	43·82	0·97
,, 2	63	67·19	0·34	59·68	0·60
,, 3	47	58·49	0·12	36·09	0·87
Barnfield plot 4–A—					
Sample 1	63	96·12	0·004	70·53	0·25
,, 2	59	93·42	0·002	88·92	0·006
,, 3	63	96·71	0·004	68·69	0·29
Barnfield plot 1–O—					
Sample 1	79	422·00	10^{-50}	84·53	0·32
,, 2	63	86·66	0·025	79·30	0·08
,, 3	63	143·81	10^{-7}	43·26	0·96

In examining a large mass of data it is more convenient to take the drop rather than the microscope field as the unit. A comparison of replicate drops also reveals any variability that may be introduced during the making of the drops. The values of χ^2 were computed for the ratios found in replicate drops from counts of 60 soil samples made by four workers over a period of some 4 years. The number of replicate drops was usually 15, but varied in different sets so that the χ^2 indices are not directly comparable. In order to collect the results into a simple diagram the values of P corresponding with each χ^2 index have been taken. Fig. 1 shows the distribution of these values, the broken line showing the distribution expected in random samples. The agreement between replicate drops is somewhat closer than would be expected from random sampling and in no set is the variability excessive. The placing of the drops on several microscope slides also enables any possible error due to variation in staining to be detected, since the ratios from replicate slides should also agree within random sampling expectations. They have always been found to do this; an example is shown in Table IV. As regards the ratios, therefore, there is experimental justification for regarding the random microscope fields as valid samples of the whole drop, and the drop as a valid sample of the suspension. Since the ratios between replicate drops have been found to agree within random sampling, the variance between such drops will depend solely on the total numbers of organisms and indigo particles counted (see Appendix) and the percentage standard error can be calculated approximately from the formula

$$S \text{ per cent.} = 100\sqrt{\frac{1}{B}+\frac{1}{I}} = s_1,$$

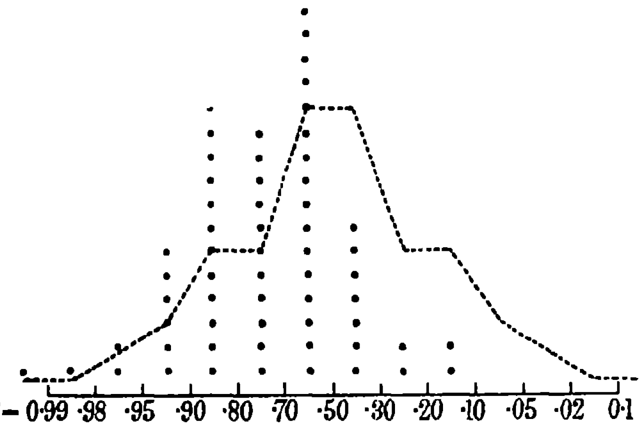

Fig. 1—Distribution of the values of P for the χ^2 indices of dispersion between replicate drop ratios from counts made by four workers from 60 soil samples derived from various sources.

where B and I are the total numbers of organisms and indigo particles. In fig. 2 the percentage standard errors that should correspond to values of $\frac{1}{B}+\frac{1}{I}$ from 0·001 to 0·015 are shown by the curve, while the crosses show actual standard errors between replicate drop ratios calculated from 20 sets of data by the empirical method based on the natural logarithms of the ratios (see Appendix). This quick method of computing the expected standard errors, from the sum of the reciprocals of B and I affords a useful means of forecasting from a preliminary count from a small number of microscope fields, how many fields must be counted to reduce the standard error between drops to a desired percentage. Thus if a preliminary count from n_1 microscope fields gives a standard error s_1, the number of fields, n_2, that must be counted to give a standard error s_2 can be obtained from the formula

$$n_2 = n_1 \left(\frac{s_1}{s_2}\right)^2,$$

such a calculation will often save counting an unnecessary number of fields. It should be noted that the standard error here involved is that between replicate drops and so does not take into account any variance due to the preparation of the films from the original samples. Any variance due to this cause, however, cannot be eliminated by counting a greater number of microscope fields.

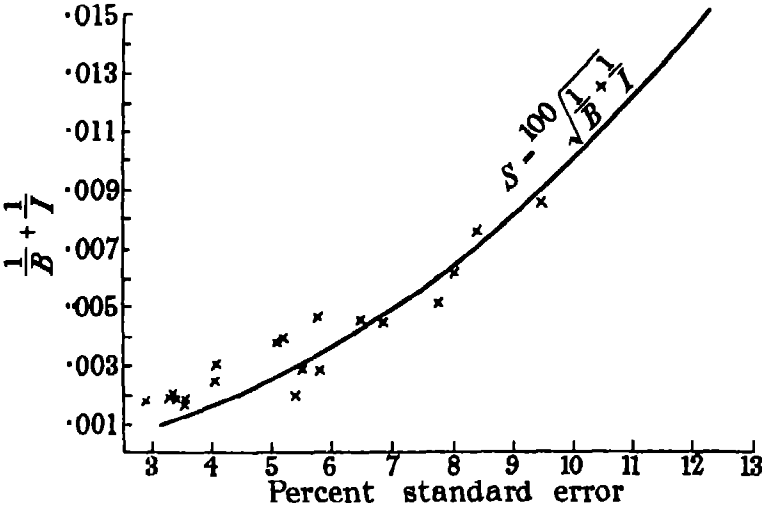

FIG. 2—Expected and observed standard errors of ratio counts plotted against $\frac{1}{B} + \frac{1}{I}$

(*b*) *Consistency of Results*—In checking the reliability of the method it is further necessary to ascertain whether the personal factor due to the worker making the counts introduces any systematic error. Such an error might not affect the random distribution of the ratios found in replicate drops. It is also necessary to test the consistency of results obtainable when the method is applied to replicate fractions of a single sample of soil. Such a test covers possible errors involved in the whole process of preparing the films from the original soil. The following experiment was made to test these two points. A sample of soil from Plot 8A of Barnfield was passed through a 3 mm sieve and, after thorough mixing, was divided into four portions. Five slides, each with five drops, were prepared from each sample. Two workers made counts

Table IV—Counts of micro-organisms by two workers from four portions of a sample of soil from Barnfield

Sample	Counts by H. G. T. Five slides of five drops counted				Counts by P. H. H. G. Five slides of five drops counted						
	Organisms	Indigo	Ratios	χ^2 between slide ratios $n = 4$	Organisms	Indigo	Ratios	χ^2 between slide ratios $n = 4$	χ^2 between workers counting $n = 25$	Mean ratios	Millions per gram of dry soil
A	1564	1024	1·527	2·9	1538	995	1·546	2·40	21·2	1·536	2758·2
B	1482	900	1·647	3·25	1498	897	1·670	3·30	12·4	1·658	2977·3
C	651	423	1·539	1·11	1298	841	1·543	1·13	21·0	1·542	2769·0
D	1420	815	1·742	4·87	1487	828	1·796	4·36	19·1	1·769	3147·8
Totals	5117	3162		12·13 $n = 16$	5821	3561		11·19 $n = 16$	73·7 $n = 100$		11652·3
Means			1·618				1·635			1·627	2913·075 ±93·08 (3·2%)

from different microscope fields from every drop. In the absence of a personal error their counts should agree as random samples. Table IV shows the numbers of organisms and of indigo particles counted and the resulting ratios obtained by each worker from each of the four portions. Column 10 shows the sums of the χ^2 indices of dispersion between the two workers' counts from individual drops of each portion. The two series of counts agreed within random sampling, no personal error being detected. Column 11 shows the mean ratios obtained from the two series of counts and the last column gives the numbers of organisms calculated per gram of dry soil, the moisture content varying slightly between the portions. The calculated numbers agreed with a standard error for the mean of 3·2% where that expected from random samples was 1·55%. The variance between portions is thus not wholly accounted for by random sampling. The extra variance was not due to the preparation of the films since replicate slides agree as random samples (see columns 5 and 9). It must thus have been present in the suspensions and probably represented a slight heterogeneity in the original soil portions.

A second experiment, with soil taken from the same plot some months later, was made to test the agreement between four portions of a single soil sample and gave the results shown in Table V. Here the standard error of the mean was 3·33%, that expected from random samples being 3·18%.

Table V—Counts of micro-organisms from four portions of a sample of soil from Barnfield

Sample	Organisms	Indigo	Ratios	χ^2 between drop ratios $n = 15$	Millions per gram of dry soil
A	477	424	1·125	13·72	2014
B	519	515	1·008	10·64	1815
C	459	419	1·109	14·02	1975
D	622	519	1·198	10·15	2135
Totals	2077	1877		48·53 $n = 60$	7939
Means			1·107		1984·75 ±66·05 (3·33%)

In the above experiments the same worker prepared the films from all the portions. It is important to ascertain whether any error is liable to be introduced by the worker's technique in preparing the films from the original soil. To test this, a sample of sifted garden soil was divided into three portions and slides were prepared and stained from each portion by a different worker. The microscope counts were all made by one worker with the results shown in Table VI. The very close agreement found shows that there was no difference due to workers' technique.

Table VI—Counts of micro-organisms from three portions of a soil sample, the preparations from each portion being made by a separate worker

Sample	Total counts of—		Ratios	χ^2 between drop ratios $n = 15$	Millions per gram
	Organisms	Indigo			
A (P. H. H. G.) .	1314	1693	0·776	4·86	1879
B (H. G. T.) . . .	994	1264	0·786	14·98	1903
C (H. L. J.) . . .	901	1168	0·771	13·4	1867
Totals	3209	4125		33·24 $n = 45$	
Means	1069·6	1375	0·778		1883 ±10·58 (0·56%)

C—"*Recovery*" *of Added Bacteria*—The correct estimation by it of a previously known number of bacteria added to sterilized soil would seem to be the final test of the ratio method. This test has been made several times. The method was to count a suspension of a pure bacterial culture on a hæmocytometer, to add a known volume of this suspension to a known quantity of autoclaved soil and at once to prepare films from this soil and estimate bacterial numbers by the ratio method. The results of five such tests are summarized in Table VII.

Table VII—"Recovery" of counted suspensions of bacteria added to sterilized soil

Organisms added	Numbers added, millions per gram	Numbers found, millions per gram	Difference, millions per gram	Percentage difference
E. coli .	8160	8220	+60	+0·74
E. coli	1160	1230	+70	+6·03
M. piltonensis	983·3	978·3	−5	−0·51
R. meliloti	880	847	−33	−3·75
R. meliloti	2080	2100	+20	+0·96

4—*Numbers of Microbial Cells in Rothamsted Soil*

The micro-organisms seen in stained films of Rothamsted soils consist for the most part of small rods and coccoid rods. Large rods found singly or in chains and *Azotobacter*-like cells are less common, but are fairly frequent in some samples. Cocci of various sizes are sometimes abundant, particularly in the Park Grass soils. Many of these are probably actinomycete spores, and the term bacteria, referring to direct counts, is used with this reservation. Fragments of actinomycete- and fungus-mycelium are numerically scarce as compared with bacterial cells. Cells which may be algæ or encysted protozoa are often seen, but the method is, of course, not adapted for preserving such large cells sufficiently well to enable them to be identified with certainty. Their numbers are, moreover, too small for counts to be of much value. Trophic forms of flagellates have occasionally been identified. Diatoms also occur though in very small numbers.

Other observers have found that the soil bacteria occur mostly as clumps or colonies. In Rothamsted soil such clumps, sometimes imbedded in the soil colloids, are at first sight a striking feature, but the actual number of cells in these aggregates is small compared with that of isolated cells, which constitute more than 80% of the total numbers.

Table VIII shows the total numbers of micro-organisms per gram found by the ratio method in some Rothamsted soils, both arable and grass land, together with the results of plate counts of the same samples made on Thornton's medium (1922). The number of cells of micro-organisms in these soils varies from 1000 to 4000 millions per gram. Taking the average volume of such a cell as 1 cubic micron, 1000 million will occupy 1 cubic millimetre and weigh about 1 milligram. An acre of Rothamsted soil to a depth of 6 inches, if taken as weighing 1·5 million pounds will thus contain from 1500 to 6000 pounds of microbial substance having 300 to 1200 pounds of dry matter with 30 to 120 pounds of nitrogen.

Table VIII—Bacterial counts from Field Soils

Plot	Manuring	p_H of soil	Total cell count millions per gram of soil	Plate count millions per gram of soil	Ratio of total count to plate count
	Barnfield (arable; continuous mangolds)				
1–O	Farmyard manure	7·6	3733	28·86	129·3
4–A	Complete minerals + ammonium sulphate	7·2	1766	15·1	117
8–O	No manure	8·0	1005	7·55	133·1
	Park Grass (permanent meadow)				
13	Farmyard manure	4·6	2395	2·25	1064·4
11–1	Complete minerals + ammonium sulphate	3·8	2403	1·35	1780·0
12	No manure	5·6	3041	7·5	405·4

There is a striking discrepancy between estimates of bacterial numbers obtained by microscope counts and by plating (see Table VIII). This is particularly great in the grass plot soils, but in this instance the low plate counts may be due to the use of a plating medium originally devised for use with neutral soils and perhaps unsuitable for the growth of the predominant organisms occurring in the acid grass plot soils. The plate counts represent an unknown fraction, probably small, of the total number of live bacteria in the soil whereas the microscope counts give the total number of live bacteria plus an unknown fraction of dead cells. It does not seem possible to determine the number of live bacteria in soil by means of the plate method because no single plating medium will support the growth of all the different groups of bacteria present, whereas if one attempts to overcome this difficulty by plating on several different media, there will be inevitable overlapping, for many types will grow on more than one medium. It can be shown that dead, or non-viable cells are included in the microscope count, since an appreciable number of cells can still be seen in stained films prepared from soil sterilized in the autoclave. Attempts to determine the number of live bacteria directly from microscope counts have therefore proved unsuccessful, although the authors have made numerous attempts to do this by various differential staining methods. It might be possible to derive an approximate estimate of the percentage of live cells included in the total count if the rate of disappearance of dead cells in the soil could be determined and were found to be approximately constant. Counts obtained by plating are derived from cells that are " live " in the sense of viable, *i.e.*, capable of multiplying on a suitable medium. There is no *a priori* reason why the number of viable cells should be more closely related, causally, to the biochemical changes in the soil than is the total number of cells, which may include many that are unable to multiply but are still able to produce chemical changes in their environment.

A survey of some of the barley plots on Hoos Field, Rothamsted, indeed, indicates a relationship between total numbers of micro-organisms and fertility. A series of soil samples were taken from 12 plots from this field on October 14, 1931, each sample being a mixture of four cores taken at random over the plot. From these samples the numbers of micro-organisms were estimated by the ratio method. Fig. 3 shows the average yield of straw for 60 to 76 years plotted against numbers of micro-organisms from 12 of these plots. There is a significant association, with a suggestion, however, that numbers of organisms are limited to about 3500 million per gram by some factor which did not similarly limit the growth of the crop.

Much more information concerning the behaviour of the total counts of micro-organisms in a field soil, will, however, be needed before their relationship to yield can be fully elucidated. For example, the sampling of the 12 plots on Hoos Field was carried out in about 1 hour, so that the time factor involved in this sampling may be important.

The plate count from soil samples taken at short intervals of time shows marked fluctuations (Thornton and Gray, 1930). It is necessary to discover how far these fluctuations also affect the total count before one can estimate the validity of counts from samples, taken at only one time. A series of samples was taken at 2-hourly intervals from a specially prepared plot of garden soil using the sampling technique described by Thornton and Gray (1930). On each occasion composite soil samples, each made by mixing six cores, were taken from the two halves of the plot and separate counts were made from each of the two samples by the ratio method. The numbers obtained are shown in fig. 4. There was a marked fall in numbers during the period of sampling and this was statistically significant.*

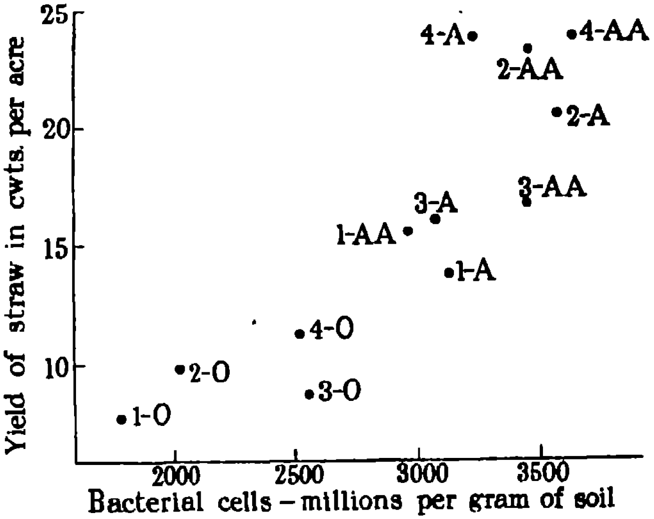

FIG. 3—Bacterial numbers by the ratio method from Hoos Field plots compared with the yield of straw. The plot number is shown against each dot. (For manurial treatments see Rothamsted annual reports.)

The fact that the numbers of micro-organisms change not only in space over a field but also in time must greatly affect the technique of comparing the micro-population of two or more field soils. Thus if the taking of the samples to be compared be not strictly simultaneous, an error may be introduced owing to changes in the numbers of organisms during the period of sampling. Moreover, a single series of samples, even if taken simultaneously, may still

* The variance between simultaneous samples was 38,107 while that between mean counts at different times was 800,147. If these variances are compared by Fisher's method (1932) the value of z is 1·52 for which P is less than 0·01.

give misleading comparisons unless the fluctuations in numbers were to run parallel in all the soils compared. The comparison of the Hoos Field plots referred to above may be subject to error from both of these causes, though the present data as to fluctuations clearly provide no means of estimating the importance of such sources of error. Fluctuations in numbers of organisms in field soils must be investigated as a preliminary to any quantitative study of the soil population in the field, since the value of all such work is dependent on a knowledge of the variance due to fluctuations. The study and analysis of the fluctuations themselves must also be of fundamental importance to the extension of our knowledge of the factors that influence the activities of the soil population.

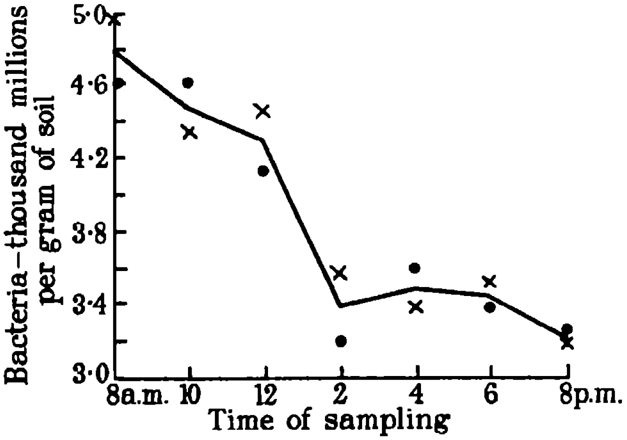

Fig. 4—Microscope counts of bacteria from soil samples taken at 2-hourly intervals. The dots and crosses represent numbers found in the two half plots.

Acknowledgment

The authors gratefully acknowledge Mr. H. L. Jensen's assistance in making some of the preparations, the counts from which are shown in Table VI.

Summary

1—There are two serious difficulties in estimating the bacterial numbers in a soil sample from the microscopic examination of stained films of that soil. The *first* is that of determining with sufficient accuracy the mass of soil in the film and the *second* that of estimating from random microscope fields the number of organisms in the film when these organisms are not distributed through it at random.

2—A technique is here described in which these difficulties are avoided, by determining, in random microscope fields from a parallel series of stained films, the *ratio* between the number of bacteria and the number of indigo particles, of which a counted suspension has previously been added to a given mass of soil. The bacterial numbers calculated from such ratios are, of course, independent of the mass of soil on the film.

3—It is found that the ratios obtained from parallel microscope fields are distributed at random, although counts of bacteria taken by themselves from the same fields are much less uniform.

4—The accuracy of the method has been tested in the following experiments :—

(*a*) The bacterial numbers in four portions of a single soil sample agreed within a standard error of 3·3%.

(*b*) The numbers found in films prepared by three workers from the same soil sample showed no significant differences.

(*c*) The numbers found by two workers independently counting different microscope fields from the same films agreed as random samples.

(*d*) Counted suspensions of bacteria added to sterilized soil were estimated with a standard error of 3·5%.

5—The bacterial numbers found in Rothamsted field soils by this method range from 1000 to 4000 millions per gram of soil.

6—Samples taken from some of the Hoos Field plots showed a relationship between the total bacterial numbers and the average yield of straw taken over a number of years.

7—Caution is at present necessary in discussing results obtained from samples taken on only a single occasion, since there is evidence of rapid changes in numbers of bacteria with time. Successive samples taken from garden soil showed significant changes in total bacterial numbers during the course of a day.

APPENDIX

By Professor R. A. FISHER, Sc.D., F.R.S.

It is important to avoid confusion between the two types of test for which χ^2 is used in this paper. If bacteria only are counted we have a single series of numbers representing the counts obtained from n' different fields, *e.g.*,

$$x_1, x_2, \ldots, x_{n'}.$$

If the different fields contained equal quantities of soil, in which after thorough mixture the bacteria were dispersed at random, it is known (Fisher, 1932, sect. 16) that the number x should constitute a sample of n' from Poisson series, such that the probability of counting just x organisms is

$$e^{-m}\frac{m^x}{x!};$$

where m is the average number in all fields. When m is not unreasonably small, *i.e.*, if it exceeds about 5 units, the homogeneity of the different fields may be easily tested by calculating what is known as the index of dispersion, defined as

$$\chi^2 = \frac{s(x-\bar{x})^2}{\bar{x}},$$

with $n' - 1$ degrees of freedom; for, on the hypothesis of homogeneity, this index will be distributed in a well-known distribution, independent of the value of m. Clearly when the values of x vary greatly χ^2 will be high, and since its distribution is known and readily available it is easy to see if its value is too high to admit of the hypothesis of homogeneity.

When both bacteria and indigo particles are counted, each field will provide two numbers, and we are concerned with the stability not of either number in itself, but of the ratio between them. This is equivalent to testing the proportionality of the entries in a $2 \times n'$ table of frequencies, for which the table of χ^2 may equally be used. If b and i stand for the numbers of bacteria and indigo particles counted in any field, and if B and I are corresponding totals for all fields, χ^2 may be written in the form (Fisher, 1932, sect. 21)

$$\frac{1}{BI} S\left\{\frac{(bI - iB)^2}{b+i}\right\},$$

where the degrees of freedom are, as before, one less than the number of fields. Thus the same distribution may be used for testing the homogeneity of the ratios as for testing that of the absolute numbers.

Where homogeneity exists the importance of obtaining clear evidence of it is twofold. In the first place, the fact that the actual discrepancies between different fields, drops, or slides are of the same magnitude as those which would arise by pure chance in sampling homogeneous material affords a guarantee that the technique employed has attained the greatest possible precision subject only to the limitation imposed by the number of fields counted. In the second place, when the precision has been thus raised to the highest possible level it depends only on the extent to which the material is counted, and can therefore be inferred from the total numbers. This is a considerable convenience in estimates of standard error, intended for use in tests of significance.

If p is the ratio $B/(I+B)$, then it is well known that, with homogeneous material, the variance of p is given by

$$V(p) = \frac{BI}{(I+B)^3};$$

from this, since I and B are both large, we may readily calculate the variance of the ratio B/I, and what is more important, that of its logarithm. For if $r = B/I$, then for small variations of p and r, dp and dr

$$\frac{dp}{p^2} = \frac{dr}{r^2},$$

whence

$$V(r) = \frac{r^4}{p^4}V(p) = \frac{(I+B)^4}{I^4}\cdot\frac{BI}{(I+B)^3} = \frac{B(I+B)}{I^3}.$$

Again if $Z = \log r$

$$dZ = \frac{1}{r}\,dr,$$

whence

$$V(Z) = \frac{1}{r^2}V(r) = \frac{I^2}{B^2}\cdot\frac{B(I+B)}{I^3} = \frac{I+B}{IB} = \frac{1}{I} + \frac{1}{B}.$$

To find the sampling variance of the natural logarithm of the ratio B/I, it is therefore only necessary to take the sum of the reciprocals of the total numbers of bacteria and indigo particles counted. This rapid and convenient test could not be relied on unless, as is shown by the χ^2 tests, practically all the existing variation is ascribable to purely random errors.

When this is not so, the values of Z derived from different drops will not agree so closely as is theoretically possible, and an empirical standard error based on actual deviations between values of Z for parallel drops should be used in tests of significance.

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INDEX TO VOL. CXV (B)

Abbie (A. A.) The projection of the forebrain on the pons and cerebellum, 504.

Allanson (M), Rowlands (I. W.) and Parkes (A. S.) Induction of fertility and pregnancy in the ancestrous ferret, 410.

Amino-acids, electrometric titration in aqueous-alcoholic solution (Neuberger), 180.

Amino derivatives of styryl and anil quinoline, antiseptic properties (Browning and others), 1.

Amino nitrogen, critique on the biological estimation (Richardson), 142.

Bacterium coli, decomposition of sodium formate by, in the presence of heavy water (Farkas and others), 373.

Basophilism, experimental pituitary (Thompson and Cushing), 88.

Bowden (F. P.) and Morris (S. D. D.) Physico-chemical studies of complex organic molecules. Part II—Absorption spectra at low temperatures, 274.

Bowden (F. P.) and Snow (C. P.) Physico-chemical studies of complex organic molecules. Part I—Monochromatic irradiation, 261.

Browning (C. H.), Cohen (J. B.), Cooper (K. E.), Ellingworth (S.), and Gulbransen (R.)—Antiseptic properties of further amino derivatives of styryl and anil quinoline, 1.

Caenis horaria (Ephemeroptera), nymph, metachronal rhythms and gill movements in relation to water flow (Eastham), 30.

Calliphora, action of X-rays on eggs (Scott), 100.

Cells, bacterial, numbers in field soils as estimated by the ratio method (Thornton and Gray), 522.

Cells, normal and malignant, factors influencing the growth in fluid culture media (Ludford), 278.

Chick embryo heart, unco-ordinated contractions caused by egg white and by alterations in the cation ratio of the medium (Murray), 380.

Clapham (P. A.) Experimental studies on the transmission of gapeworm (*Syngamus trachea*) by earthworms, 18.

Cohen (J. B.) *See* Browning and others.

Colour adaptation phenomena (Wright), 49.

Cooper (K. E.) *See* Browning and others.

Cowan (S. L.) The action of potassium and other ions on the injury potential and action current in *Maia* nerve, 216.

Cushing (H.) *See* Thompson and Cushing.

Das (B. K.) The habits and structure of *Pseudapocryptes lanceolatus*, a fish in the first stages of structural adaptation to aerial respiration, 422.

Earthworms, experimental studies on the transmission of gapeworm (*Syngamus trachea*) (Clapham), 18.

Eastham (L. E. S.) Metachronal rhythms and gill movements of the nymph of *Caenis horaria*, 30.

Eccles (J. C.) and Hoff (H. E.) The rhythm of the heart beat. I—Location, action potential, and electrical excitability of the pacemaker, 307.

Eccles (J. C.) and Hoff (H. E.) The rhythm of the heart beat. II—Disturbance of rhythm produced by late premature beats, 327.

Eccles (J. C.) and Hoff (H. E.) The rhythm of the heart beat. III—Disturbance of rhythm produced by early premature beats, 352.

Ellingworth (S.) *See* Browning and others.

Farkas (A.), Farkas (L.) and Yudkin (J.) The decomposition of sodium formate by *Bacterium coli* in the presence of heavy water, 373.

Farkas (L.) *See* Farkas and others.

Ferret, anœstrous, induction of fertility and pregnancy (Allanson and others), 410.

Ferret, effect of absence of light on the breeding season (Hill and Parkes), 14.

Flying, high, limit when breathing oxygen (Hill), 298.

Forebrain, projection on the pons and cerebellum (Abbie), 504.

Fox (H. M.) The oxygen to iron ratio of oxychlorocruorin and the total quantity of oxygen carried by the pigment in *Spirographis*, 368.

Gapeworm, experimental studies on the transmission by earthworms (Clapham), 18.

Gill movements, in relation to water flow, of the nymph of *Cænis horaria* (Eastham), 30.

Gray (P. H. H.) *See* Thornton and Gray.

Gulbransen (R.) *See* Browning and others.

Hæmoglobin, kinetics, IV, V, VI and VII (Roughton), 451, 464, 473, 495.

Heart beat, rhythm I, II and III (Eccles and Hoff), 307, 327, 352.

Heart of chick embryo, unco-ordinated contraction (Murray), 380.

Hill (A. V.) Nerve heat production as a physiological response to excitation, 200.

Hill (Sir Leonard) The limit of high flying when breathing oxygen, 298.

Hill (M.) and Parkes (A. S.) Effect of absence of light on the breeding season of the ferret, 14.

Hill (R. T.) and Parkes (A. S.) Hypophysectomy of birds. I—Technique, with a note on results, 402.

Hoff (H. E.) *See* Eccles and Hoff.

Hypophysectomy of birds (Hill and Parkes), 402.

Irradiation, monochromatic (Bowden and Snow), 261.

Ludford (R. J.) Factors influencing the growth of normal and malignant cells in fluid culture media, 278.

Molecules, complex organic, physico-chemical studies, I and II (Bowden and Snow and Bowden and Morris), 261, 274.

Mongolism, relative aetiological importance of birth order and maternal age (Penrose), 431.

Morris (S. D. D.) *See* Bowden and Morris.

Murray (P. D. F.) Unco-ordinated contractions caused by egg white and by alterations in the cation ratio of the medium in the heart of the chick embryo *in vitro*, 380.

Nerve heat production as a physiological response to excitation (Hill), 200.

Nerve, *Maia*, action of potassium and other ions on the injury potential and action current (Cowan), 216.

Neuberger (A.) Electrometric titration of amino-acids in aqueous-alcoholic solution, 180.

Oxychlorocruorin, oxygen to iron ratio (Fox), 368.